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13. ABSTRACT ***(Maximum 200 Words)***

A steadily growing body of evidence indicates that mouse and human mammary cancers present as clonal diseases that depend upon genetic alterations both for their initiation and progression. It is considered likely, but not proven, that the only cells of any tissue able to pass on genetic aberrations to their progeny are those that retain the capacity for cell division and that do not become terminally differentiated. This hypothesis has renewed interest in the biology and characteristics of stem cells in many tissues, with the notion that such information will be useful for the prevention, detection and therapy of the disease. In recent years it has been shown that even tissues like brain, heart, and skeletal muscle, that were previously thought to have no stem cell compartment do, in fact, have a population of stem cells. Research on the nature and characteristics of hematopoietic stem cells has produced stem cell markers and a large body of information on mechanisms of oncogenesis in myelocytes and lymphocytes. These advances have made it apparent that we need a thorough knowledge of normal growth and development and thus of the stem cell population of the mammary epithelium to understand and treat breast cancer. If the stem cell compartment plays an important role in the transition of mammary epithelium from a normal to a malignant cell population, determining the dynamics, regulation, and biology of these cells could be of considerable value to the prevention and therapy of cancer.

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Introduction

A Stem Cell Origin for Tumors

Several lines of evidence suggest that human cancers may have their origin in stem cell populations (49). Stem cells possess several characteristics that are considered signatures for the tumor phenotype. Stem cells are mobile (1;26) (32) which allows them to migrate from the bloodstream or an injection site and join the parenchyma of virtually any tissue. It is now known that even tissues like brain, heart, and skeletal muscle, which were previously thought to have no stem cell compartment, do have a population of stem cells. All of these tissues can receive stem cells from bone marrow that differentiate into cells with molecular signatures characteristic for that tissue (17;22;23;32). Such mobility has been confirmed in humans in whom donor cells were detected in the liver after receiving bone marrow transplants (51).

Another important stem cell property is the plasticity that enables them to respond not only to the environment of their native tissue, but also to the specific growth stimuli peculiar to any tissue. For instance, transplanted bone marrow cells have been found to produce neurons in brain (17;29), liver cells (33), and muscle cells (22). Also, stem cells of muscle differentiate to brain neurons (23) and blood cells (28), and stem cells from brain migrate to bone marrow (2) and give rise to myocytes. Showing even greater potential adult mouse neural stem cells can be incorporated into the embryos of chick and mouse to give rise to cells from all three embryonic germ layers. Stem cells also possess the properties of self-renewal and unlimited replicative capacity. Stem cells persist through life and the numbers of gene alterations required for transformation appear to accumulate in cells that persist for long periods of time in a single state of differentiation. This makes them better candidates for sites of sporadic tumor formation by loss of tumor suppressor gene function since the more differentiated progenitor cells are believed to have too small a window before terminal differentiation to allow the accumulation of enough mutations to become tumor cells. The increased incidence of tumors with age also suggests that mutations accumulate in populations of persistent cells. Recent investigations on the mechanisms of oncogenesis in myelocytes and lymphocytes has produced evidence for an origination point in hematopoietic stem cells (12;37).

Clonal Nature of Mouse and human Mammary Tumors

A steadily growing body of evidence indicates that mouse and human mammary cancers present as clonal diseases that depend upon genetic alterations for their initiation and progression. Several studies have shown that mouse mammary tumors and hyperplasias are the clonal

progeny of a single mutated cell (5;13;25;31;45;50), implying that they may arise from division-competent cells within the epithelium (42). Inactivated X-chromosome analysis has shown that in human mammary epithelium entire terminal lobulo-ductal units (TLDUs) and patches of mammary ductal epithelium are the clonal progeny of a single stem cell that maintains the ductal patch or TLDU (15;53). This has brought new interest to the possibility that mammary stem cells may be the initiating point for breast cancer. Information about the properties of the stem and progenitor cell populations will be useful for the prevention and detection of breast cancer and for new therapeutic drug discovery. Already, new drugs that target stem cell properties in leukemias and other cancers are in the late phase clinical trials (16;35;41;52).

This laboratory works with two transgenic mouse models of human mammary cancer that over-express oncogenic proteins believed to be fundamental in breast tumorigenesis. These are the c-myc over-expressing and the TGFα over-expressing transgenic mice. In the c-myc mouse the defining characteristics of the tumor are metastasis and chromosomal aberrations associated with genomic destabilization (19;34;54). The tumors of the TGFα mouse are pregnancy dependent and the oncogene causes impaired apoptotic function so that post-lactational involution does not occur (47). Growth and apoptosis are functions of the stem and progenitor cell populations in all tissues beginning in embryonic and fetal development. We are using these two mouse models to discover how growth and apoptosis are regulated in these populations and how over-expression of c-myc and TGFα act to disrupt this regulation and initiate tumorigenesis. Each proliferative cell type in a tissue has its own set of properties that defines its contribution to the tissue (4;6;11). Every cell that inherits chromosomal instability and/or a loss of apoptotic control from its predecessor may, due to its place in the lineage, have properties that require different anticancer therapy from those that preceded it. As such altered progenitor cells expand and differentiate they add to the steadily increasing population of differentiated malfunctioning tumor cells.

Stem Cells in Mammary Epithelium

Evidence for mammary epithelial stem cells has grown in the last thirty years. Autochthonous serial transplantation of mammary gland has demonstrated that the progeny of the cells in a fragment of mammary epithelium can repopulate new mammary fat pads for at least seven generations (27). Repopulation and clonagenicity studies indicate that, as in the hematopoietic system (48) and liver (36), only a single stem cell may provide enough potential to completely repopulate mouse mammary gland (43), producing both ducts and lobules(14;30). Furthermore, a fragment from any mammary epithelial structure can be transplanted to generate a new fully lactating mammary gland. Thus, both the ducts and the terminal end buds contain cells with full

regenerative power. However, due largely to the lack of knowledge about specific molecular markers, neither stem nor progenitor cells can be identified in isolated of mammary epithelial cells. Despite this limitation, two undifferentiated, morphologically distinct, division-competent mammary epithelial precursors can be microscopically visualized in mouse (46), rat (7;8), cow (18), sheep (18) and human (20) mammary epithelium. Recently, morphometric analysis in the rat showed that one of these morphotypes represents two separate populations that appear to have different functions (7). We believe that this very undifferentiated morphotype represents both a stem and a primary progenitor cell population. Without molecular markers these cells can only be recognized by morphological characteristics. Therefore, Chepko and Smith (9) assessed division-competence in a specific cell type by determining whether or not it is ever observed to contain mitotic chromosomes. Then they classified all 5 cellular phenotypes in the epithelium by eight morphological criteria.. These characteristics are: mitotic competence, staining of nuclear and cytoplasmic matrix, nuclear morphology, amount and size of cytoplasmic organelles, cell size, cell shape, cell number, location relative to the ductal lumen and basement membrane, and grouping relative to each other and to other morphotypes. Using these criteria a working designation was developed for each cell type. The two most undifferentiated cells were named Small Light Cells (SLC) and Undifferentiated Large Light Cells (ULLC) (7;8). Phenotypes intermediate between the two undifferentiated morphotypes, and between them and the functionally differentiated secretory and myoepithelial cells have been observed. A morphometric analysis of the five populations determined that there are actually three division-competent cells in rat mammary gland (6). Together the morphological observations and morphometric data suggest that there is a stem cell hierarchy in the mammary gland, and that there may be a unidirectional genealogy from SLC (the least differentiated cells), through the ULLC, to the fully differentiated secretory and myoepithelial cells. This evidence is supported by the recent discovery by Pechoux et al.. (40) that human myoepithelial cells do not give rise to lumenal epithelial cells, but that a subset of lumenal epithelial cells divide to produce myoepithelial cells. Both division-competent morphotypes have been observed in mouse mammary gland hyperplasias (7) and in c-myc mouse mammary tumors (44). Chepko (8) and Chepko and Smith (7) have proposed such a genealogy, based on the morphometric studies.

There are, as yet, no molecular markers for mammary epithelial stem or progenitor cells, because their immunohistochemical differentiation is confounded by the affinity of mouse mammary stroma for the chromogen 3,3'-diaminobenzidine tetrahydrochloride (DAB). This produces enough background staining to obscure the faint signal expected in rare cells. The mammary epithelium of mouse, sheep, cow, and human were shown to contain the same morphotypes, and the ULLC at least is known to be division-competent in human(21). The morphological recognition of these cells is complex and depends heavily on a specific fixation, dehydration and staining protocol for the tissue, and it does not allow specific identification of the function of each division-competent cell type.

Research Directions

In this grant we propose to determine if and how the stem cell compartment may be involved in the transition from normal to neoplastic mammary epithelium in our transgenic mouse models. We propose to:

- Work out an effective staining method and tissue preparation protocol for use with confocal or conventional light microscopy that will allow recognition and quantification of the two division-competent morphotypes in normal mouse mammary epithelium.
- Determine the sizes of the three different division-competent cell populations in normal mouse epithelium.
- Compare the normal results to those obtained for the mammary epithelia of the *c-myc* and *TGFα* transgenic mouse models of mammary hyperplasia and tumorigenesis breast tumors both in the hyperplastic stages and in palpable tumors.

Basic Methods

- We propose to use confocal microscopy in order to take advantage of the optical sectioning ability, multiple imaging, and image integration capacities of the instrument. This will produce the most informative data in the shortest amount of time and will optimize the significant amount of manual labor and number of man-hours that such a project demands.
- We will compare the *in vivo* cell cycle profiles of the mammary epithelial cells in each transgenic strain with that of the non-transgenic FVB mouse.
- We also propose to collaborate with a biostatistician to develop a mathematical model with which to statistically analyze the large amount of data that this project will generate.

We expect the results of this novel proposal to demonstrate if and how stem cells are involved in passing on characteristics that cause neoplastic transformation and progression. We will also determine if there are differences in the regulation of the division-competent population compartments in the transgenic tumor models compared to those of the normal non-transgenic mouse.

Necessity for Modification in Aims IB and IIA

Research in the past two years on the nature and characteristics of hematopoietic and other stem cells has produced molecular markers that permit isolation of cells enriched for

stem or progenitor cell properties. Recent work in the rat hippocampus also shows that the flk-1 /VEGF2R/KDR is not only a marker for endothelial progenitor cells but for neural (39) and hematopoietic stem cells (38) (3) as well. This discovery along with that that stem cells and early progenitors of many origins have enough plasticity to differentiate into virtually any tissue opens the possibility, and increases the probability, that they may all carry similar markers. If this is possible we may now be able to use both frozen section fuorescence / confocal microscopy and cytometry to search for stem/ progenitor cell markers in mammary epithelium. In a revised statement of work in this report we propose such a modification of Specific Aims IB and IIA. *We feel it is timely and of pressing importance to make this modification at this time.*

Body

Results

Specific Aim IA

The BrDU labeling study proposed in AIM I was converted into a PCNA staining and cell count due to the failure of the Alzet osmotic pumps used to deliver BrDU. However, we used the material to perform the proposed population study by staining mammary epithelial tissue sections for Proliferating Cell Nuclear Antigen (PCNA). We have collected the data from 5 of the 6 mice in the study for a comparative cell type composition analysis of ducts, lobules and overall mammary epithelium for the control FVB, and mutant c-MYC and TGFα mouse models of breast cancer. The uncounted mouse is the other FVB (control) mouse.
The PCNA positive (PCNA+) vs PCNA negative (PCNA-) cell counts are providing data for cell cycle analysis of the 5 cell populations in FVB, c-MYC and TGFα genotypes. The data collected to date are presented here.

Overall Epithelial Composition:

- The cell populations are present in roughly the same proportions in the overall normal (FVB) mammary epithelium as found in the rat (10) (Table 2). There was no significant difference in population sizes between the normal and transgenic animals when the entire epithelium was considered. However when the counts in ducts were compared with those in lobules the ULLC (presumptive secondary progenitors) in lobules was a significantly larger population in the FVB and TGFα animals, but was the same size as in the ducts of the c-MYC mice (Fig 2). The other small but significant difference was that the size of the SLC population in TGFα ducts was only half the size of that in either ducts or lobules of the other two genotypes.
- The ULLC account for 15% of the FVB mammary epithelial cells, DLLC 8%, LDC 60% and MYO 11% (Fig. 2). ULLC and DLLC were not counted separately in the rat and they comprised a combined population of 9% of the epithelium. When combined in the mouse they represent a much larger portion of mammary epithelial cells: 23%.

PCNA Staining:

- There is a pronounced difference between ducts and lobules in the proportion of PCNA+ cells in nearly every population, with the lobules having more proliferative activity than the ducts (Fig 2).
- LDC comprise the largest population in the epithelium. They and the MYOs are ultrastructurally the most differentiated of the 5 cell types and they both contain a lower proportion of PCNA+ cells (Fig.).

- Analysis of PCNA-positive cell populations in all three genotypes.
  An overall difference in percent PCNA-positive cells between ducts and lobules was seen.

Percent of Cells Staining Positive for PCNA		
Genotype	Ducts	Lobules
FVB	9%	47%
MYC	7%	10%
TGFα	2%	19%

The presence of 3 Terminal End Buds in the FVB mouse accounts for the high label in FVB lobules. They were included as positive counts in normal growth

Morphological Observations

Of the 15 animals used for this experiment only 5 produced epithelial preparations in which the 4 morphotypes could reliably identified. Two other protocols of fixation and dehydration were tried in order to increase the number of useable specimens. Finally, communication with other mammary gland biologists suggested that such inconsistency of morphology is common and has lead us to question whether it is related to the stage of estrus at the time of fixation. To address this problem in the future we are currently fixing mammary gland, ovaries and uterus from 13 normal FVB mice from which we have collected daily pap smears for 2 weeks. If morphology is affected by the estrus stage, we will choose a stage that provides uniformly good morphology and use animals at that stage for the rest of the experiments for this grant.

The cytology of the cell types in the FVB control epithelium was comparable to that of the rat, and the cytology of the TGFα epithelium was similar to that of the FVB. However, the hyperplastic areas of the c-myc epithelium contained cells that although they stained lightly with hematoxylin and varied in size, were difficult to impossible to categorize (Fig. 4) according to the criteria below.

PCNA Staining and Cell Identification

Some cells of every morphotype stained positive for PCNA, and chromatin conformations consistent with G0, G2, and G1/S, but not mitosis showed both PCNA positive and PCNA negative staining. PCNA positive staining ranged from very deep to very pale for every cell type and for all chromatin conformations and was interpreted as indicating that the cell was probably either in the cell cycle or finishing its final cycle. (However, the presence of PCNA in differentiated cells may indicate they are differentiating and leaving the cell cycle). As in the rat and all previous observations in the mouse only SLC and ULLC were seen in mitosis. Mitosis was rare in all three genotypes.

Morphometry

Cell counts are complete for 5 mice: 1 FVB, 2 c-MYC, and 2 TGFα. Total cell counts for each genotype were 7,388 for FVB, 7,166 for Myc, and 7247 for TGFα. Cell counts for the final FVB are underway. Figure 2 presents the results of the population breakdown by cell type for each genotype.

Statistics

Statistical Methods. The total cell counts for each cell type and the number of cells labeled within each category were combined to create the percent of cells labeled within each cell type. Three-way analyses of variance (ANOVA's) with fixed effects were performed to determine any differences in the percents of cells labeled among genotype, cell type, and category. The use of these percents was selected to meet the assumption of data normality required for the test.

Any effect with a p-value less than 0.05 is considered significant in terms of effect of PCNA labeling.

Statistics Results: Table 1 presents the ANOVA results, which indicate that the genotype and the cell category, ducts vs. lobules, significantly differ in terms of percent of PCNA+ cells ($p=0.026$ and $p=0.002$, respectively). Specifically, FVB had the highest PCNA+ staining rate, followed by TGFa and then Myc. Lobules are also more likely to be PCNA+ compared to ducts, regardless of genotype and cell type. Cell type was not a significant effect with this sample size, but the trend confirms our initial hypotheses, indicating that MYO cells do in fact have the lowest percentage of label and ULLC and DLLC have the highest. This is consistent among all genotypes (Figure 1). Due to small numbers, these results need to be verified with the new counts currently underway.

Development of Criteria for Identifying Mammary Epithelial Cell Types

Collection of Images for cell counts: This was done using the Olympus AH2 Vanox microscope with a Toshiba 3-chip color CCD low light level camera. Images were collected from paraffin sections of normal FVB mouse mammary epithelium using in ImagePro under oil immersion at 630-1000X magnification. They were compared to the electron microscope images used to make the original cell type classifications(7).

Digital images were collected with an Olympus AH2 Vanox microscope with a Toshiba 3-chip color CCD low light level camera. Full images of all sections were collected at 60X using an automated motorized stage encoder and stage controller for image tiling with a Stage-Pro Stage Control Module. An image of each section in its entirety was captured, filed and printed out. Epithelial elements were chosen for analysis based on their cellular integrity and goodness of stain as evaluated at 400X. Each was then circled on the print of the full section, and files of images of each circled structure were collected through two or three focal planes at 630X magnification. In this way a complete record of each epithelial structure and its location was kept through each serial or consecutive section. A final folder containing more than 2 gigabytes of images was filed on CDROMs.

Epithelial structures were classified as ducts when they were associated with no other epithelial structures. They were classified as lobules if they were part of a group of other similar sized epithelial structures or if they were buds from a larger duct that was sectioned longitudinally. Cells in TEBs were included in the lobule count because the animal was more than 3 months old and they were located at the limit of the fat pad. Using the above criteria cells in each image were scored PCNA+ or PCNA- and counted according to morphotype. ImagePro 3.0 image analysis software was employed and the manual tag feature was used to count the five morphotypes. Data for each mouse were collected automatically into Excel files, and counts for lobular epithelium were collected and analyzed separately from counts for ductal epithelium.

Criteria for Identifying Epithelial Cell Types:

Large Dark Cell (LDC) Fig. 4A:

Contacts the lumen.
Round or irregularly shaped nucleus.
Densely stained heterochromatin and nuclear matrix.
Contains a nucleolus.
Cuboidal / polygonal shape.
Cytoplasm stains a definite blue/gray.

Differentiated Large Light Cell (DLLC) Fig. 4B:

Contacts the lumen. Larger than an LDC.

Round nucleus with densely stained heterochromatin. May be oblong and with axis parallel to basement membrane.
Nuclear matrix may stain lightly.
May contain a nucleolus.
Pale-staining cytoplasm.
Cuboidal / polygonal shape or oblong like a myoepithelial cell. (NOT a round cell.)
Undifferentiated Large Light Cell (ULLC) Fig. 4 C,D,E:
May or may not contact the lumen.
No nucleolus.
Large, round, often pale-staining nucleus with no apparent heterochromatin.
Nucleus may be large, but slightly oblong with the longest axis parallel to the basement membrane. These cells do not contact the lumen.
Large, pale staining cytoplasm with rounded or irregular cell boundaries. (These are not polygonal cells.) Cytoplasm frequently bulges into the lumen.
Myoepithelial Cell (MYO) Fig. 4B :
Never contacts the lumen, but sits above the basement membrane.
Elongate nuclei lying parallel with the basement membrane and that bulge toward the epithelium. Elongate nuclei bulging away from the epithelium were considered fibroblasts and excluded from the count.
Elongated irregular cytoplasm that is often invisible without actin-specific stain.
Nucleus contains heterochromatin and matrix stains deep blue.
Small Light Cell (SLC) Fig. 4 A,F :
NEVER contacts the lumen.
Two sizes: very small, and almost the size of an LDC. The very small SLC are rarer.
Nucleus contains densely stained heterochromatin.
Nucleus appears as elongate or small and round.
Cell is either round or irregularly shaped.
Cytoplasm to nucleus ratio somewhat more than 1:1.

Any cell that could not be classified according to the above criteria due to shrinkage, overstaining, unusual morphology associated with hyperplasia, section thickness, or angle of cut through the epithelium was counted but classified as Unknown (UK).

Cytometry
Cytometry of FVB Female ME through the estrus cycle:
No difference in cell cycle profile with stage of estrus cycle.
97% cells in G_1/G_0
1.6% cells in G_2/M
0.8% in S.

Cytometry of c-myc tumors.
87% cells in G1/G0
5-6% cells in G2/M
8% cells in S

REVISED STATEMENT OF WORK
Specific Aim IA ***Months 12-24***

Work out the aspects of sectioning, staining, and light microscopy and data collection techniques. **Finished.**

Technical Assistant. Mating of FVB non-transgenic mice (8 weeks from mating to post-puberty). **Finished.**

Examination of non-transgenic mammary glands: determine estrus staging, BrDu injection, collect, fix, process, and section the glands. **Nearly finished, but used anti-PCNA.**

Section staining, microscopy, data collection. **Finished.**

Biostatistician. Mathematical model development. **(Specific Aim IV)** **Finished.**

Isolate and culture non-transgenic mammary epithelial cells to confluence, label with BrDU and transplant into epithelium-free mammary fat pads of 34 d non-transgenic female mice. Allow to grow for 3, 5, 7, 9, 12, 24, 36, days and 6 weeks. **Not begun**

Collect and prepare tissue from task 6 for section staining. **Finished.**

Section staining, microscopy, data collection. **Finished.**

Technical Assistant. Mating of *c-myc* mice (8 weeks from mating to post-puberty) **(Specific Aim II).** Genotyping of offspring (1 week) **Not begun.**

Biostatistician. Statistical data analysis data. **Nearly Finished.**

Specific Aims IB and IIA – Modified (See Original Aims in Appendix) ***Months 13-24***

A. Use markers that have been used to differentiate stem cells and committed progenitors in some other tissues search for progenitor-limiting markers for stem cells of normal (FVB) mammary epithelium. Since VEGF2R/Flk-1/KDR is now known to be present on hematopoietic (3;38) and neural stem/progenitor cells (39), we propose to use antibodies against it on frozen sections (fluorescent and/or confocal microscopy) to determine if it may be present on the SLC or ULLC. If we find it, we will confirm it in paraffin sections. We will also try other possible markers such as c-kit and sca-1, which, although long used to study and isolate hematopoietic stem cells have recently been shown to be present on murine skeletal muscle stem cells (28). We will also use the Hoecsht 33342 efflux assay (24;28) for stem cells.

B. Use the markers found to isolate subpopulations of cells for determining their respective sizes in the epithelium, and correlating marked cells with the established morphologies.

C. Use newly discovered markers to viably isolate and culture stem/progenitor cell populations for labeling with BrDU and transplantation into the mammary fat pad and allowing different growth times (variable numbers of estrus cycles) to determine the roles of different populations in mammary gland development. We will try to assay this by staining with an anti-BrDU antibody and counting cells that have and have not lost BrDU label in the time elapsed. We will use the morphological criteria established in the past year and knowledge gained from Specific Aim IIB. Development of a statistical model may be necessary for this as proposed before.

D. Repeat Specific Aim IIC for c-myc and TGFα mammary epithelial cells.

We believe that defining the stem/progenitor cell kinetics of the different populations in normal and oncogene over-expressing epithelium that we will learn if any of the different cell types play special roles in normal growth, hyperplasia and tumorigenesis.

We anticipate that the results of this revised aim may cause revision of Aim III. We will await the results to determine how it can best be modified.

Specific Aim III ***Year 3***

1. *Technical Assistant.* Mating of *TGFα* transgenic mice, and *c-myc* × *TFGα* transgenic mice, pregnancy, and growth to post-puberty of offspring. Genotype offspring.

2. Examination of *TGFα* and *c-myc* mammary tumors from *TGFα* and *c-myc* transgenic mice: determine estrus staging BrDu injection, collect, fix, process, and section the glands.

3. Section staining, microscopy, data collection.

4. Isolate and culture mammary epithelial cells from *TGFα* and *c-myc* tumors to confluence, label with BrDU and transplant into epithelium-free mammary fat pads of 34 d non-transgenic female mice. Allow to grow for 3, 5, 7, 9, 12, 24, 36, days and 6 weeks.

5. Collect and prepare tissue for sectioning.

6. Section staining, microscopy, data collection from tissue.

7. *Biostatistician.* Statistical analysis of data.

8. Isolate mammary epithelial cells from *TGFα* and *c-myc* glands, prepare for cell cycle analysis (FACS) by the flow cytometry core.

9. Examination of mammary epithelium of young (5 8week old animals) *TGFα/c-myc* preneoplastic females and males. Determine estrus staging (females), BrDu injection, collect, fix, process, and section the glands.
10. Collect and prepare tissue from task 32 for sectioning.

11. Section staining, microscopy, data collection from tissue.

12. *Biostatistician.* Statistical analysis of data and integration of all data.

13. Perform interphase FISH analysis on paraffin sections mammary gland of all three transgenic animals plus the non-transgenic.

Key Research Accomplishments

Aims IA and II ***Specific Aim IA***: Using anti-BrDU-labeled mouse mammary epithelium to visualize division-competent cellular mophotypes and a fluorescent antibody against specific cytokeratins to view the differentiated morphotypes, we will develop a method to catalog large amounts of morphological data by confocal, or conventional light microscopy, and image analysis.

- We worked out a cell type identification procedure for paraffin sections by light microscopy consistent with that published for electron microscopy(10).

- We developed a method of data management, and ImagePro was used to manually count cells and save the file for each duct and lobule to Excel.
- We carried out a comparison of population size, and percent of the population in the cell cycle for each mammary epithelial cell population in normal FVB mice, and 2 tumor models: c-Myc and TGFα transgenic mice.

- We compared the percent of each population in the cell cycle between ducts and lobules.

Specific Aim IB and IIA: Determine in non-transgenic mice the number of proliferating and non-proliferating cells in the population of each cellular morphotype for each stage of the estrus cycle. Determine the frequency of symmetric and asymmetric mitoses in each stage of the estrus cycle.

- We determined the proportion of cycling cells (PCNA positive) and non-cycling cells (PCNA negative) in each population in transgenic (Aim IIA) and non-transgenic (AIM IB) mouse mammary epithelium.

We would like to modify Specific Aims IB and IIB: -(See Revised statement of work for details.)

Antibodies for the Vascular Endothelial Growth Factor Receptor 2/ FLK-1/KDR that is common to stem cells of neurons in adult rat hippocampus, bone marrow and endothelium have become available for mouse. We believe that to reduce the labor-intensive nature of this work it is important at this time to search for useful markers for the stem and progenitor cells of the mammary epithelium. After we have markers to differentiate the stem and committed progenitors we will have better tools to determine the proportion of each morphotype that is proliferating. We hope that defining the stem/progenitor cell kinetics of the different populations in normal and oncogene over-expressing epithelium that we will help us learn the roles of the different cell types. We will do this by cytometry of isolated epithelial cells and by fluorsescence and/or confocal microscopy in frozen sections of mammary epithelium of mice whose estrus stages have been determined both by pap smear and ovarian examination.

Specific Aim IIB: Determine whether the *in vivo* cell cycle profiles of *c-myc, TGFα,* and double transgenic mouse mammary glands differ from that of non-transgenic glands.

- We have collected cell cycle profiles for the four estrus states for the FVB mouse and for c-myc tumors.

Reportable Outcomes

Poster Presentation: 10th Anniversary Celebration of NIH Office of Research in Women's Health Sept 10-11, 2000 "Stem and Progenitor Cell Populations in Mouse Mammary Gland", G. Chepko, B. K. Vonderhaar, and R. Dickson.

Review: "Mammary Epithelial Stem Cells". 2001. Gilbert H. Smith and Gloria Chepko, Microscopy Research and Technique 52:190-203.

Conclusions

The primary question this grant seeks to address is whether the lesion(s) that cause cancer occurs in a stem cell population. The data gained in the first year did not inform this question. However, we have determined the relative sizes of the different cell populations in normal plus two models of hyperplastic epithelia (Fig. 3). The c-myc mouse is considered a model for human ductal carcinoma, and the TGFα mouse models lobular tumorigenesis. No significant variation in population size among the genotypes can be detected with a sample size of 7200 cells per genotype. This may indicate that regulation of growth and apoptosis in these populations may indeed be key in tumorigenesis.

We showed that within genotypes the SLC is uniformly the smallest population comprising about 3% in ducts and lobules. This is consistent with that found in rat (Chepko) (Table 2). However, contrary to the study for rat the DLLC and ULLC were counted separately and the population of DLLC (presumed differentiating population of ULLC) is about half the size of the ULLC in both ducts and lobules. The LDC is consistently the largest population in both rats and mice ranging for mice from 55 to 70% between ducts and lobules. As a population myoepithelial cells appear to be about the same size in lobules as in ducts. The sizes of the ULLC and the myoepithelial cell populations are nearly similar.

If the remaining cell counts confirm this data, there may be important implications for our understanding of mammary tumorigenesis. First, these results do not support a model whereby excessive or unrestrained proliferation leads to mammary tumors. Rather, the anti-apoptosis effects of *TGFα* suggested by Smith et al (47), and the chromosome destabilizing effects of *c-myc*(19;34) may be critical features in these models. Second, the data suggest that growth in the normal and transgenic epithelia occurs in the lobular regions in the ULLC/DLLC populations. The ULLC are the presumed to be the expanding population of progenitors, so the data supports this model. Ultrastructural studies (8;10;20;21) suggest that the ULLC give rise to DLLCL which will become lumenal cells (LDC) and to DLLCM which are in the process of becoming myoepithelial cells (MYO). There is no apparent difference in the sizes of either of these populations between ducts and lobules, or between transgenic and normal epithelium (Fig. 2 A and B). Also, since both transgenics appear to follow the same trend in cell proliferation (Fig. 1), the rate of expansion in the progenitor cell (ULLC) compartment could be disregulated through either of the two mechanisms that the oncogenes affect.

The F statistic indicates that a sample size of 5-10 times the size that used for each genotype would be necessary to determine a cell population specific effect. In view of this a more productive effort would be to use a battery of prospective stem cell markers to identify stem and progenitor cell populations in frozen sections. This has been successful in adult rat hippocampus and helped to define interactions between the stem cell niche and angiogenesis (39). Once identified the markers could be used on isolated mammary epithelial cells to perform viable cytometric cell separation and quantification. Each population could then be studied both in primary cell culture and as thymidine analog-marked, *in vivo* transplants into epithelium-free mammary fat pads to determine its properties and role(s) in epithelial development, hyperplasia and tumorigenesis. Once such tools have been created therapeutic drug studies can be pursued with a deeper understanding.

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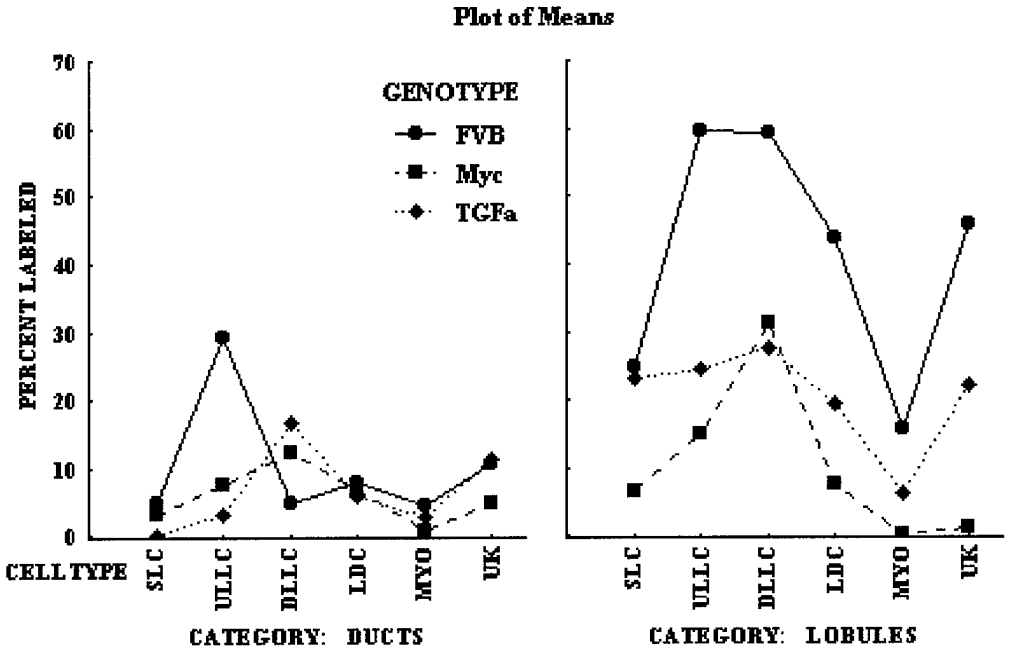

Figure 1. Plot of Mean Percent Labeled for Each Cell Type, Within Genotype, Within Category

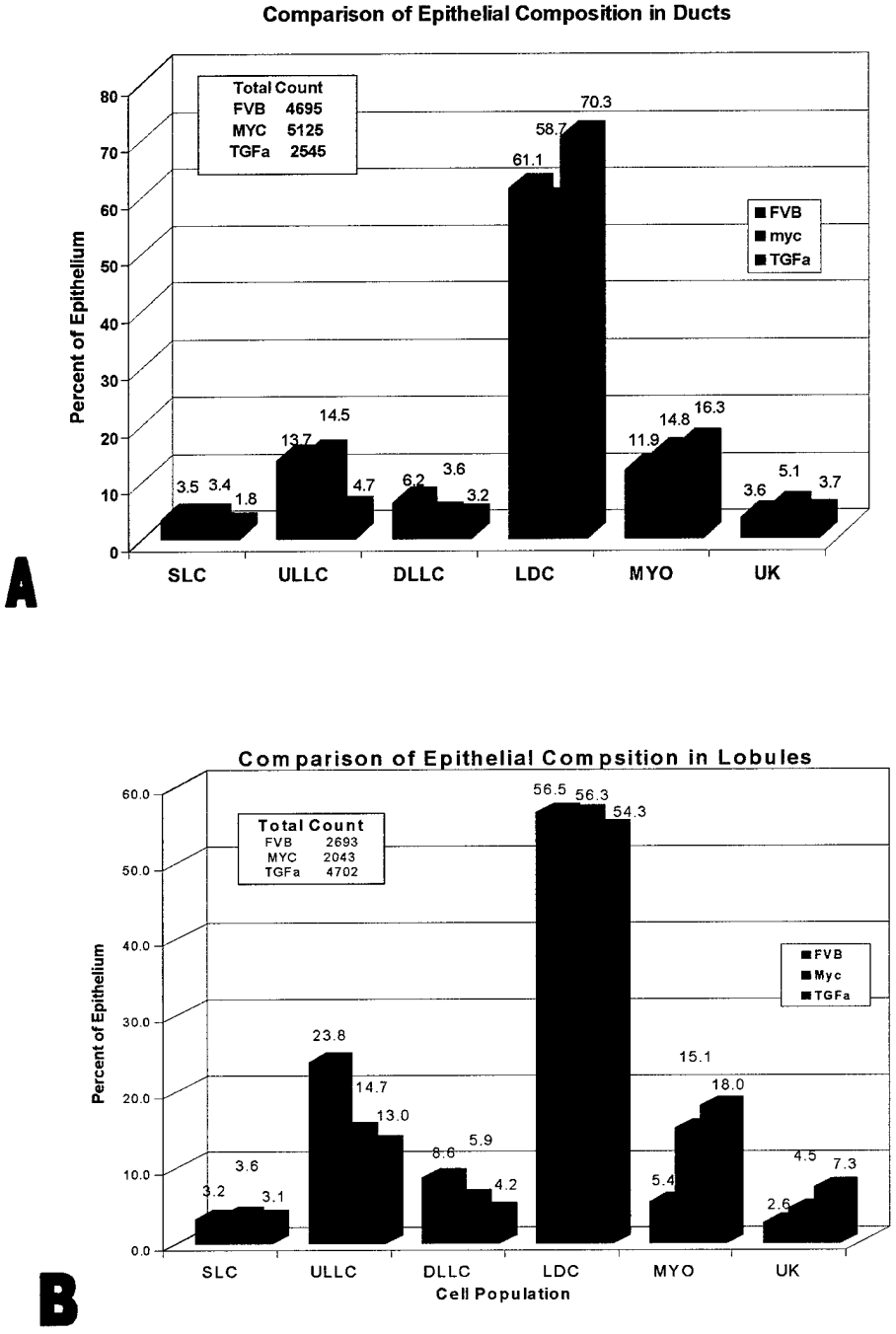

Figure 2. Comparison of the sizes of the cell populations of mammary epithelial ducts (**A**) and lobules (**B**) in FVB (green), *c-myc* (red) and *TGFα* (blue) mice.

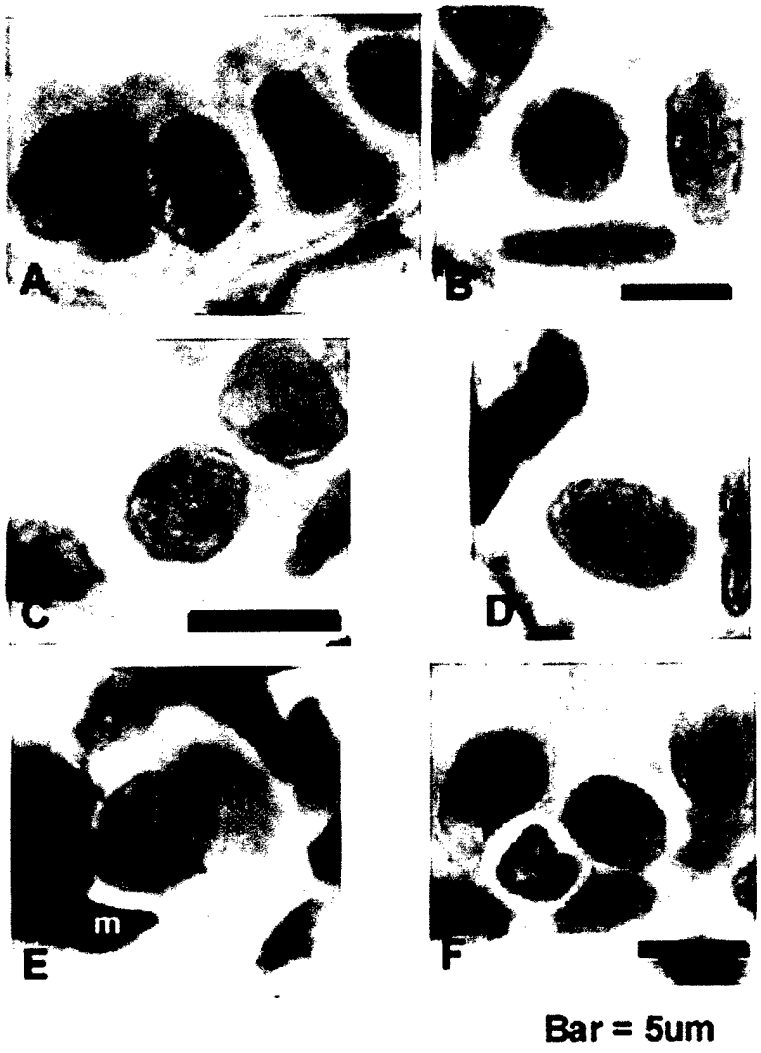

Figure 3. Representative pictures of the five cellular morphotypes that compose rodent mammary epithelium. L, LDC; s, SLC; D, DLLC; m, myoepithelial cell; u, ULLC.

Figure 4. Cytology of a hyperplastic duct in c-myc epithelium stained with anti-PCNA antibody. The cell types are very difficult to categorize according to the criteria developed. Those that are unidentifiable are labeled "uk", those that have characteristics that place them predominately into a category have a question mark (?) following their label. Lu, ductal lumen; L, LDC; m, MYO; f, fibroblast; D, DLLC; u, ULLC.

Table 1. Analysis of Variance results for Percent of Cells Labeled with PCNA

Effect	Degrees of Freedom	Mean Square	F-Statistic*	P-value
Genotype (G)	2	1269.109	4.276	0.026**
Cell Type (CT)	5	523.477	1.764	0.159
Category (CG)	1	3628.394	12.226	0.002**
G/CT Interaction	10	91.350	0.308	0.972
G/CG Interaction	2	719.902	2.426	0.110
CT/CG Interaction	5	131.442	0.443	0.814
G/CT/CG Interaction	10	59.365	0.200	0.994
Error	24	296.774		

F-Statistic = (MS Effect/DF Effect)/(MS Error/DF Error); Genotype = FVB, Myc, TGFa; Cell Type = SLC, ULLC, DLLC, LDC, MYO, UNK; Category = Ducts, Lobules.

Table 2. Cell Populations in Rat Mammary Epithelium

Stage	SLC	LLC	Other (LDC)	Myo*
Virgin	3%	8.5%	68.5%	20%
Pregnancy	3%	4%	73%	20%
Lactation	3%	4%	73%	20%
Involution	3%	4%	73%	20%
Total Count	111	141		
Total Cells Counted		3552		

* Myoepithelial cells were estimated and the estimate was correlated with that of two other experienced mammary gland biologists

Original Aims

Hypothesis I: There is a unidirectional genealogy of mammary epithelial cells, beginning with Small Light Cells, that gives rise to Undifferentiated Large Light Cells. The latter through symmetric mitosis can produce secretory cells, or they can divide asymmetrically to give rise a myoepithelial cell and a secretory cell. Neither the secretory cells nor the myoepithelial cells in normal gland can undergo cell division.

Rationale: Although two division-competent cellular morphotypes have been identified in the mammary epithelia of rodents, cows, sheep and humans, there has been little investigation of the actual lineage of the five known morphotypes of the mammary epithelium. However, this information is fundamental to our understanding of the biology of normal and pathological mammary epithelium. Furthermore, regulation of asymmetric versus symmetric mitosis during growth of the epithelium may be an important mechanism in the maintenance of the balance between differentiated and undifferentiated division-competent populations.

Specific Aim IA: Using anti-BrDU-labeled mouse mammary epithelium to visualize division-competent cellular mophotypes and a fluorescent antibody against specific cytokeratins to view the differentiated morphotypes, we will develop a method to catalog large amounts of morphological data by confocal, or conventional light microscopy, and image analysis.

We have found that hematoxylin, which can be used to visualize all mammary epithelial morphotypes with conventional microscopy is not sufficiently fluorescent to use in the confocal microscope as a counterstain to fluorescently labeled BrDU. We first propose to develop a counterstain that is more effective in this respect, with our back-up strategy to utilize immunohistochemistry with conventional light microscopy. We will then develop an efficient way to catalogue and manage the large amount of morphological data to be collected and analyzed on the Image Analyzer.

Specific Aim IB: Determine in non-transgenic mice the number of proliferating and non-proliferating cells in the population of each cellular morphotype for each stage of the estrus cycle. Determine the frequency of symmetric and asymmetric mitoses in each stage of the estrus cycle.

1. Loss of label in stem cells. Stage estrus cycles of 6-8 week female non-transgeneic mice by vaginal smear. Inject BrDU in PBS directly into the mammary fat pad at the estimated edge of the growing epithelium after opening and reflecting the skin. Inject at proestrus, estrus, diestrus, and met-estrus. Remove the left number 4 mammary gland under anesthesia 1 hour after injection, and prepare according to the information we obtain from Specific Aim IA. Determine the stage of estrus for each animal just before sacrifice by vaginal smear. Sacrifice animals at 6hr, and 1, 2, 3, and 4 days post-labeling. Prepare glands as established in

2. Stain serial sections with anti-BrDU antibodies, and examine by microscopy as established in Specific Aim IA. Determine relative proportions of each morphotype, labeled and unlabelled. Digitally capture contiguous epithelium in successive serial sections. Use the Optimus 5.2 Image Analyzer to establish a standard of staining intensity that will demonstrate progressive loss of label with successive cell divisions. Count the number of each morphotype and measure the amount of label each cell contains at each

time point. Count the number of labeled and unlabeled symmetric and asymmetric mitoses in both SLC and ULLC. Analyze for progressive loss of label in each morphotype versus the number of unlabeled cells in each morphotype (see hypothetical data set – TableII). Evaluate data using mathematical model to be established in Specific Aim IV.

3. Retention of label in stem cells. Culture primary mouse mammary cells in BrDU-containing medium to confluence, and transplant into cleared fat pads of 34-day-old non transgenic mice. Allow to grow for 3, 5, 7, 9, 12, 24, and 36 days, and 6 weeks; fix and prepare tissue as in IB(1) and IB(2). Examine and quantify as in Specific Aim IB2. Analyze for progressive loss of label in BrDU-labeled cells as in IIB2. Evaluate data with mathematical model to be developed in Specific Aim IV.

Hypothesis II: Preneoplastic mammary epithelium transgenic for overexpression of *c-myc*, *TGFα*, or both transgenes may either exhibit altered proportions or numbers of the division-competent morphotypes compared to the normal animals. They may include cells that normally do not divide at all, and there may be an alteration in the balance of asymmetric and symmetric mitoses. Furthermore, if there are alterations in the division-competent populations, the transgenic glands may exhibit different cell cycle profiles compared to the non-transgenic epithelia.

Rationale: Mammary glands of *c-myc* transgenic mice develop tumors at about 10 months of age, while mammary tumors in mice transgenic for *TGFα* are associated with pregnancy. However, both the latency and frequency are significantly increased for the mice bitransgenic for both genes. These growth characteristics imply that the population dynamics in division-competent cells of mammary epithelium predisposed to neoplasia may differ from the normal epithelium.

Specific Aim IIA: Determine the number of proliferating and non-proliferating cells in the population of each cellular morphotype in the preneoplastic *c-myc, TGFα, and c-myc-TGFα* transgenic mouse mammary glands. Determine the frequency of symmetric and asymmetric mitoses.

1. Loss of label in stem cells. Use the data from Specific Aim I to inform the choice of estrus stage for labeling with BrDU. Determine estrus stage of 6-8 week-old female *c-myc*, *TGFα*, and *c-myc-TGFα* mice by vaginal smear. Inject BrDU in PBS directly into the mammary fat pad and as remove the left number 4 mammary gland in IA. Prepare as established in IB. Determine the stage of estrus for each animal just before sacrifice by vaginal smear. Sacrifice animals at 6hr, and 1, 2, 3, and 4 days post-labeling. Prepare glands as established in IA. Process tissues, and analyze digitally captured micrographs for loss of label in BrDU-labeled cells as in IB. Evaluate data using mathematical model to be developed in Specific Aim IV.

2. Retention of label in stem cells. Culture primary mouse mammary cells from adult female *c-myc* and *TGFα* mice in BrDU-containing medium (3 ug/ml). Grow to confluence, and transplant into cleared fat pads of 34-day-old normal mice. Allow to grow for 3, 5, 7, 9, 12, 24, and 36 days, and 6 weeks. Sacrifice animals, prepare tissue and examine microscopically. Examine and quantify as in IB2. Analyze for loss of label in BrDU-labeled cells as in IB(2). Here we expect to see retention of label in a very small population of stem cells that are permanently resident. Evaluate data using the mathematical model to be developed in Specific Aim IV.

Specific Aim IIB: Determine whether the *in vivo* cell cycle profiles of *c-myc*, *TGFα*, and double transgenic mouse mammary glands differ from that of non-transgenic glands.

1. Perform FACS analysis of isolated mammary epithelial cells of adult, *c-myc*, *TGFα*, and double transgenic mice.
2. Perform interphase FISH to determine presence and degree of aneuploidy in transgenic mammary gland for *c-myc* or *TGFα*.

Hypothesis III: The division-competent cell populations in the neoplastic epithelia induced by the *c-myc* and *TGFα* oncogenes may be different in size, proportion and cell cycle status from the normal mammary gland. Furthermore, the balance between symmetric and asymmetric cell divisions may be strikingly altered and the tumors may exhibit different cell cycle profiles than the non-transgenic epithelia and transgenic preneoplastic epithelia.

Specific Aim IIIA: Determine the number of proliferating and non-proliferating cells in the population of each cellular morphotype in the palpable mammary tumors of *c-myc*, *TGFα*, and *c-myc-TGFα* mice. Determine the frequency of symmetric and asymmetric mitoses.

1. Loss of label in stem cells. Use the stage of estrus established in IB for labeling with BrDU. Stage the period of estrus cycle of 6-8 week-old female *c-myc*, *TGFα*, and *c-myc-TGFα* mice by vaginal smear. Inject BrDU in PBS directly into the mammary fat pad as in IB. Remove the left number 4 mammary gland under anesthesia 1 hour after injection, and prepare as established in IB. Determine the stage of estrus for each animal just before sacrifice by vaginal smear. Sacrifice animals at 6hr, and 1, 2, 3, and 4 days post-labeling. Prepare glands as established in IA. Stain serial or near serial sections with anti-BrDU antibodies and examine microscopically. Count the number of each morphotype and the amount of label each contains at each time point. Count labeled and unlabeled morphotypes and analyze as in IA. Evaluate data using mathematical model to be developed in Specific Aim IV.
2. Retention of label in stem cells. Culture primary mouse mammary epithelial cells from adult female *c-myc* and *TGFα* mice in BrDU-containing medium (3 ug/ml). Grow to confluence, and transplant into cleared fat pads of 34-day-old normal mice. Allow to grow for 3, 5,7,9.12, 24, and 36 days, and 6 weeks, and prepare tissue as in IA. Analyze for loss of label in BrDU labeled cells as in I(2) . Evaluate data using mathematical model to be developed in Specific Aim IV.

Specific Aim IIIB: Determine whether the in vivo cell cycle profiles of epithelial cells in, *c-myc*, *TGFα*, and double transgenic mouse mammary tumors differ from that in normal or preneoplastic transgenic epithelium.

1. Perform FACS analysis of mammary epithelial cells isolated from, *c-myc*, *TGFα*, and double transgenic tumors.
2. Perform interpase FISH to determine if a certain proportion of particular morphotypes are aneuploid in the transgenic tumors.

Specific Aim IV: Develop a mathematical model for evaluating the data sets in normal mammary epithelium (Table II), and determining the process of population dynamics. Use this tool to compare the data between the normal, preneoplastic, and tumorous epithelia to determine the role(s) of the division-competent cells in both normal epithelium and tumorigenesis.

Data from half of the mice in Specific Aim I will be used to develop a novel statistical model to describe and test the transitional stages of cell division and differentiation. Existing techniques from linear models, (43,44) branching processes, (45-47) and other methods which become apparent through research will be explored. Once a model is developed and meets statistical and scientific acceptability, it will be tested with the other half of the data. Necessary adjustments will be made until the model sufficiently and reliably explains the division and differentiation process of the five mammary epithelial morphotypes. Table 2 indicates the format of the data. The model must estimate the frequency of cell division (both symmetric and asymmetric) for each morphotype, and address the following assertions: i) a proportion of stem cells are persistent, meaning that they rarely divide and their daughter cells are stem cells; ii) the proportion of persistent stem cells differs in hyperplastic and tumor epithelium from normal epithelium, (Hypotheses I, II, and III); iii) cell division differences will exist among the three cell types (Hypotheses I, II, and III); and iv) SLCs may divide into SLCs or ULLCs (or MYOs) and ULLCs may divide into ULLCs, DLLCs, or MYOs, but the reverse is not true.

At Day 1 the 25 SLC's that labelled at 6 hours have divided once, and half of them have joined the ULLC compartment. As the estrus cycle progresses on days 2, 3, and 4 SLC divide and differentiate, moving to the ULLC compartment and ULLC divide and move into either the DLLC which later become LDC's or MYO's. The statistical model will estimate the time for each transition. The Reverse experiment (Specific Aims IA(3) and IIA(2))for ex vivo labeling and transplantation would show an initial labeling of all SLC and with progressive time a retention of label in the 0.5% that may be primary stem cells.

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Mammary Epithelial Stem Cells

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KEY WORDS epithelial stem cells; asymmetric mitosis; symmetric mitosis; mammary gland; morphogenesis; progenitors

ABSTRACT It has recently been shown that the progeny from a single cell may comprise the epithelial population of a fully developed lactating mammary outgrowth in mice. Serial transplantation of epithelial fragments from this clonally derived gland demonstrates that the subsequently generated outgrowths are also comprised of progeny from the original antecedent. All epithelial cell types were found to be present within these clonal normal populations including luminal, myoepithelial, ductal, and lobule-committed epithelial progenitors and fully competent mammary epithelial stem cells. These observations demonstrate the presence of multipotent tissue-specific epithelial stem cells among the parenchyma of the murine mammary gland. Similarly, genetic analysis of contiguous portions of individual human mammary ducts within the same breast indicates their clonal derivation. Here, we discuss the properties, location, division-potential, senescence, and plasticity associated with mammary epithelial stem cells and present the developing evidence for their presence in human breast and their important role in the risk for breast cancer development. Further, we review the present morphologic and genetic evidence for the characterization of specific stem cell markers and lineage-limited progenitor cells in human and rodent mammary epithelium.
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INTRODUCTION

At birth, the female mouse possesses mammary glands composed of branched epithelial cords that contain lumina, i.e., a system of branching hollow epithelial tubes that open at the nipple (Hogg et al., 1983). Little subsequent mammary growth and development occurs until the onset of puberty, when the immature gland grows rapidly to produce the tree-like pattern of ducts, upon which the secretory alveolar lobules form during pregnancy. During this period of growth and invasion of the mammary fat pad by the mammary duct system, beginning at approximately 3 weeks of age and continuing until approximately 12 weeks of age in virgin females, intense mitotic activity occurs within the terminal mammary end buds. It is in these structures where the first evidence for mammary epithelial stem cells was discovered. At the tip of growing terminal end buds the dividing, undifferentiated cap cells can adopt alternate epithelial cell fates (Williams and Daniel, 1983). If cap cells enter the epithelial population of the end bud body, they become part of the luminal cell population. Alternatively, if they migrate laterally along the outermost layer of the subtending duct in contact with the fibrous stroma, their fate becomes myoepithelial. This appears to be the result of the action of extrinsic factors on cap cells. Their adoption of alternate cell fates and their high mitotic activity led the authors to suggest that cap cells are multipotent mammary epithelial cells. However, cap cells and terminal end buds disappear when the mammary fat pad is completely filled by the mammary ductal tree and never appear again during the lifetime of the mouse. In addition, all portions of the mammary epithelium (and not just the ductal tips) retain the capacity to regenerate the entire mammary epithelial structure upon transplantation into mammary fat pads, demonstrating that cap cells are not the only multipotent mammary epithelial cells.

The action of mammary stem cells and their mitotic progeny is fundamental to normal mammary growth, differentiation, and regeneration in successive cycles of pregnancy, lactation, and involution. An important feature of the mammary gland is the regenerative capacity of its epithelium, which is demonstrated upon successive reproductive cycles. During pregnancy in the mouse, a massive increase (25–27-fold) in the number of mammary epithelial cells occurs within the mammary fat pad (Kordon and Smith, 1998; Nicoll and Tucker, 1965). Following lactation, this massive epithelial population is subsequently reduced to a number very close to that in the nulliparous fat pad through apoptotic cell death during a process called involution. Understanding normal mammary growth, differentiation, and subsequent remodeling is essential to understanding the aberrant growth that results in mammary tumors.

An early marker for tissue-specific stem cells was based upon ultrastructural morphology and the absence of organellar features indicative of functional differentiation. Most of our knowledge of mammary stem cells comes from cell and tissue transplantation studies in the mouse and from histological examina-

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tions of postnatal developmental stages of murine mammary gland at the light and electron microscope levels. The human mammary epithelium shows corresponding ultrastructural cytology (Ferguson, 1985, 1988)and current evidence from rodent models suggests that mammary epithelium contains more than one type of multipotent epithelial precursor (Chepko and Smith, 1997; Kordon and Smith, 1998; Smith, 1996).

The prevailing view regarding stem cells is that they are cells with the capacity for prolonged if not unlimited self-renewal and can produce at least one type of fully differentiated descendant. Often, between the stem cell and its differentiated progeny, there may be an intermediate population of committed progenitors with a restricted differentiation potential and a limited capacity for self-renewal. Tissue-specific stem cells have the capacity both to self-renew and to generate all the differentiated progeny represented in the tissue. While this definition applies to all stem cells, the potential of an organ-specific stem cell differs depending on the tissue and the kind of population dynamics required to maintain tissue function.

New data about organ-specific stem cells is rapidly accumulating in diverse organ systems, e.g., liver (Michalopoulos and DeFrances, 1997), brain (Levison and Goldman, 1997; McKay, 1997; Morrison et al., 1997), skin (Jones, 1997), lung (Emura, 1997), and muscle (Ferrari et al., 1998). Research on stem cells in blood (Boll, 1980), intestine (Cosentino et al., 1996), cerebellum (Snyder et al., 1992), and retina (Turner and Cepko, 1987; Turner et al., 1990) support the conclusion that more than one type of multipotent cell may be present in an organ system (Morrison et al., 1997). In all of these organ systems, it has been shown that the immediate progeny of a stem cell are division-competent. Therefore, in all these tissues there exists a hierarchy of division-competent cells: primary tissue-specific stem cells, which can produce all the cell types comprising the tissue including new stem cells, progenitor cells, which are oligopotent, i.e., produce only a subset of the cell types of an organ, and lineage-committed progeny that may reproduce only equivalent cells. Self-renewing multi-lineage progenitors are considered stem cells for practical purposes because they can be long lived and produce many cell types in a tissue (Morrison et al., 1997).

Mammary epithelial stem cells possess special properties that allow them to persist in the tissue and to propagate new cells without differentiating. One strategy used to increase their reproductive lifetime is to generate cell diversity through the creation of surrogate multipotent progenitors. Tissue-renewing stem cells vary relative to the number of downstream progenitor cell sets they produce. For skin, the keratinocyte stem cell has been shown to have one set of progenitors downstream (Jones and Watt, 1993; Jones, 1997) called transit amplifying cells. In the mammary gland, multipotent cells have been demonstrated in the mouse and in the human (Pechoux et al., 1999; Smalley et al., 1999; Smith, 1996; Stingl et al., 1998). Smith (1996) presented evidence for the presence of three distinct multipotent epithelial cells in the mouse mammary gland: one capable of producing all of the epithelial cell types present in fully functional lactating gland and two downstream progenitors that were limited in their ability to produce either secretory lobulogenesis or branching ductal morphogenesis in pregnant hosts. Subsequently, Kordon and Smith (1998) established that all three multipotent epithelial cell types in the mouse mammary gland arise from a single antecedent.

STEM CELL MARKERS

Cell-specific markers have been found for some tissue-specific stem cells, e.g., c-kit for hematopoetic (Sakabe et al., 1998a,b), and liver stem cells (Michalopoulos and DeFrances 1997). Cd34 is a marker for lymphocyte precursors (Sakabe et al., 1998b), and the $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins for epidermis (Jones and Watt, 1993). Nestin, microtubule-associated-protein-2 (map-2), and glial fibrillary acidic protein (gfap) are markers for neural cell precursors (Meltzer et al., 1998). Attempts to identify mammary lineage specific markers have yielded CALLA and MUC1 as surface markers for myoepithelial and luminal mammary epithelial cells, respectively, in the human breast (Pechoux et al., 1999; Stingl et al., 1998). Several recent studies have demonstrated that the multipotent cells in mammary epithelium reside within the luminal cell population in human and mouse (Pechoux et al., 1999; Smalley et al., 1999). However, no specific molecular signature for mammary epithelial stem cells was revealed. Smith and Medina (1988) presented an earlier marker that held promise for identifying mammary stem cells in the ultrastructural description of mitotic cells in mammary epithelial explants. These investigators noticed that mouse mammary explants, like mammary epithelium in situ, contained pale or light-staining cells, and that it was only these cells that entered mitosis when mammary explants were cultured.

In the absence of a distinct biochemical marker, Chepko and Smith (1997) reasoned that it would be possible to analyze light cells in the electron microscope utilizing their ultrastructural features to distinguish them from other mammary epithelial cells. Applying the basic defining features of multipotent stem cells, namely division-competence, mitotic quiescence, asymmetric mitosis, symmetric mitosis, and a possible undifferentiated cytology, we determined what images we might expect to see in electron micrographs. We realized that we would observe only a few mitotic cells, but that we could expect to examine them for images that may suggest mitotic competence (presence of mitotic chromosomes), symmetric or asymmetric cell division, such as the orientation of metaphase chromosomes relative to the plane of the basement membrane. Further, since the pale-staining (stem) cells were morphologically different, their appearance in side-by-side pairs or in one-above-the-other pairs (relative to the basement membrane) might suggest a recent mitosis. In addition to pairs, other informative images would be of cells that were morphologically intermediate between a primitive and differentiated morphology based on the number, type, and development of cytoplasmic organelles. Cells may be evaluated for cytological differentiation with respect to their organelle content and distribution, e.g., cells differentiated toward a secretory function might contain specific secretory products, such as milk protein granules or micelles, which have been ultrastructurally and immunologically defined

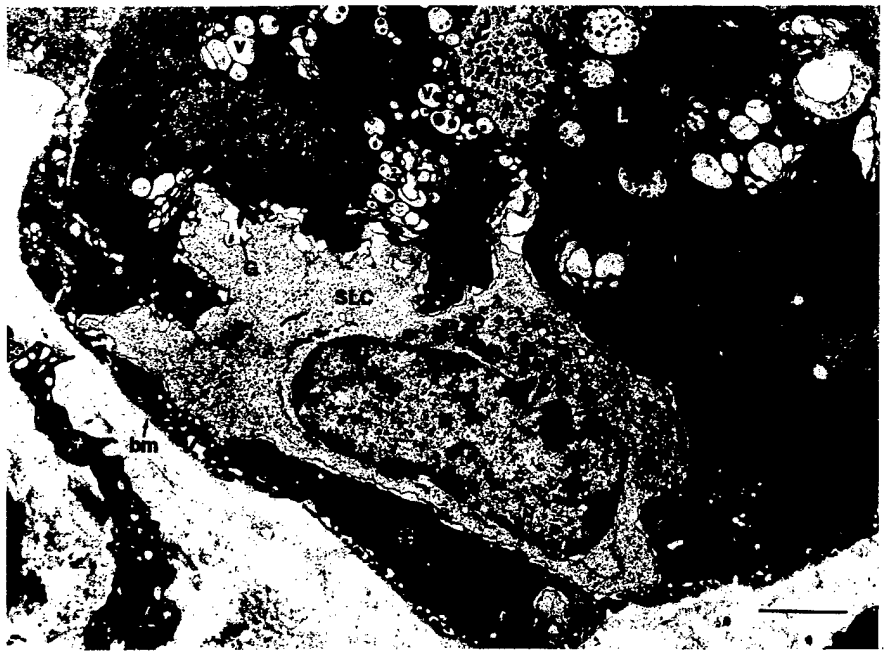

Fig. 1. A solitary, pale-staining, SLC in 6 day lactating rat mammary gland, showing the characteristic morphology of these cells. Notice the sparse organelles clustered close to its nucleus (N), the pale-staining nucleoplasm and cytoplasm, the heterochromatin (H) in the indented nucleus (arrowhead), cytoplasmic processes, and the fact that it does not reach the epithelial lumen (Lu). ER, endoplasmic reticulum; m, mitochondria; LDC, large dark cell; v, golgi vesicle; L, lipid droplet; bm, basement membrane. Bar = 1.0 μm.

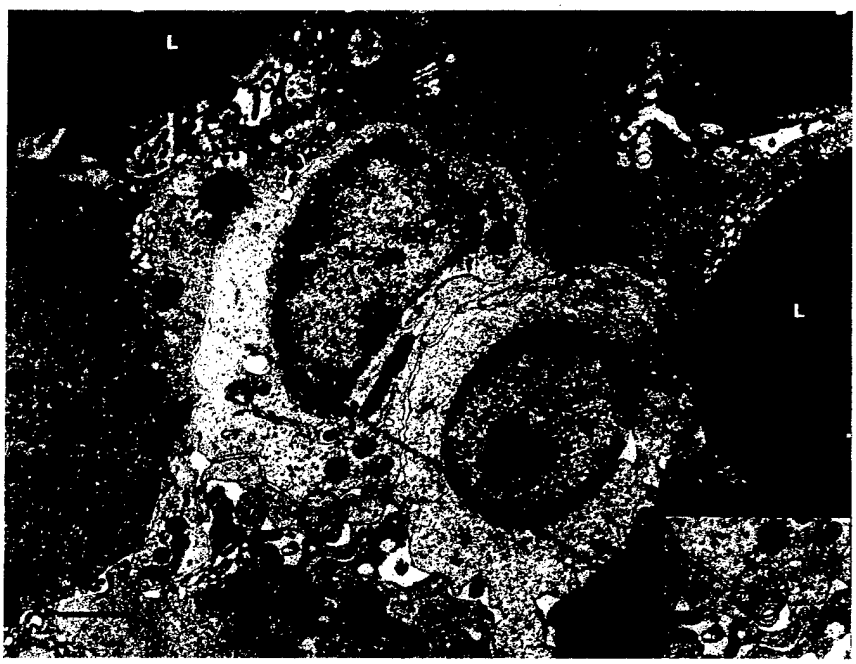

Fig. 2. SLC in like pairs. A typical homogeneous, side-by-side pair of SLC in 2–3 hour lactating rat mammary gland flanked on either side by Large Dark Cells (LDC). The nuclei are heterochromatic, organelles are sparse, and the cytoplasm extends into folded processes. The cells do not reach the lumen and are linked to each other and neighboring cells by non-junctional focal contacts (circles and **inset**). Their appearance in pairs infers that SLC may produce selfsame daughter cells when they divide. m, mitochondria; bm, basement membrane; LDC, large dark cell; L, lipid droplet; v, Golgi vesicle; My, myoepithelial cell; Lu, lumen. Bar = 1.0 μm. Inset: ×7,500.

(Hogan and Smith, 1982; Shimmin, 1964). In addition, the presence and number of intracellular lipid droplets, the extent and distribution of Golgi vesicles, and rough endoplasmic reticulum (RER) attest to the degree of functional secretory differentiation of a mammary epithelial cell. These features are characteristically well developed in the luminal cells of active lactating mammary gland. Myoepithelial cells are flattened, elongated cells located at the basal surface of the epithelium, and their prominent cytoplasmic feature is the presence of many myofibrils and the absence of RER or lipid droplets.

In a retrospective analysis of light and electron micrographs, we performed a careful and detailed scrutiny of mammary epithelium to determine the range of morphological features among the cell types than had previously been reported. The samples evaluated included mouse mammary explants, pregnant and lactating mouse mammary glands, and rat mammary gland from 17 stages of development beginning with nulliparous through pregnancy, lactation, and involution (Chepko and Smith 1997; Smith and Vonderhaar 1981; Smith et al., 1984; Vonderhaar and Smith 1982). From this analysis, we were able to expand the number of cell

Table 1. Structural and Functional Characteristics of Mammary Cell Morphotypes[a]

Characteristic	Small Light Cell	Large Light Cells		Large Dark Cell
		Undifferentiated	Differentiated	
Percent Total Cells	~3%	~5% (9% in virgin)		~70-75% (with myoepithelial cells comprising the difference)
Location/ Polarity	No lumenal contact/ None.	May or may not contact lumen/ Baso-Luminal	Contacts lumen/ Baso-luminal	Contacts lumen/ Baso-luminal
Shape	Amoeboid	Round or gourd-like	Polygonal; epthelioid character	Polygonal; epthelioid character
Nuclear Morphology	Small; pale nucleoplasm with hetero-chromatin	Very large; round; pale, fibrillar, nucleoplasm; no heterochromatin	Large; round; pale nucleoplasm, with heterochromatin	Medium; oval, round, or irregular; light to dark nucleoplasm with heterchromatin
Cytoplasmic Characteristics	Pale with few organelles	Pale with some RER, secretory granules, small lipid droplets	Pale; well developed golgi and RER; secretory granules and lipid droplets	Deeply stained; well developed golgi and RER; secretory granules and lipid droplets
Mitosis	yes	yes	?	?
Grouping	1. Singles 2. Like pairs 3. Mixed pairs 4. Clusters	1. Singles 2. Like pairs 3. Mixed pairs 4. Clusters	1. Singles 2. Like pairs 3. Large arrays	Comprised most areas of epithelium
Function	Division com-petent; mixed population of hypothesized stem cells and I°progenitor cells	Division competent; probable II°progenitor cell; makes some milk products	Synthesis and secretion of proteins and lipids; continues to differentiate	Synthesis and secretion of proteins and lipids; fully differentiated secretory cell

[a]Excluding myoepithelial cells

types in the epithelium from two secretory (or luminal) and myoepithelial cells to five distinguishable structural phenotypes or morphotypes. Our observations support the conclusion that only the undifferentiated (light) cells enter mitosis. The undifferentiated cells occur in two easily recognized forms : small (~8 μ) and large (15–20 μ) (Figs.1, 2). Once we determined that the darker cells, namely secretory and myoepithelial cells, at no time contained mitotic chromosomes, we rationalized that they were terminally differentiated and out of the cell cycle. Using all of the above features, we were able to develop a more detailed description of the epithelial subtypes that comprise the mammary epithelium.

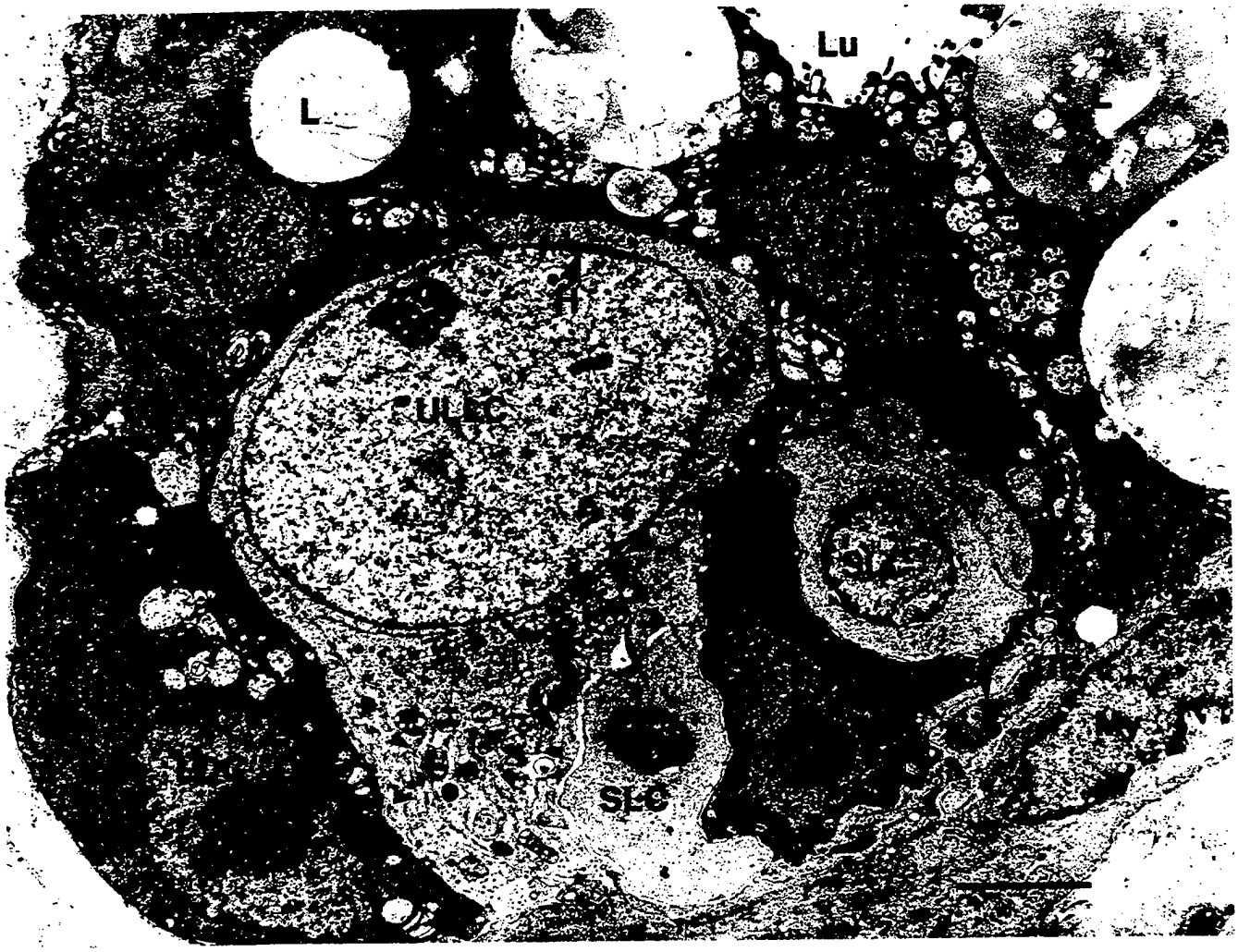

Fig. 3. Mixed Pairs of SLC. A side-by-side mixed pair of light cells consisting of an SLC and an Undifferentiated Large Light Cell (ULLC) in 2–3 hour lactating rat mammary gland. The presence of mixed pairs infers that SLC may also produce ULLC. The narrow base of the ULLC rests on the luminally oriented surface of a myoepithelial cell (My), but the apical portion of the cell does not contact the lumen (Lu). The large, apically located nucleus has very little heterochromatin (H), and the cytoplasm contains scattered mitochondria (m) and RER (arrowheads). To the right of the ULLC is an SLC surrounded by a large intercellular gap that is characteristic of SLC. To the right of the large dark cell (LDC) that flanks the mixed pair on its right is a second SLC in close contact with a portion of a second light cell (lp). Below the LDC is a portion of a ULLC (UP) containing a lipid droplet (L), some organelles and RER (arrowheads). v, Golgi vesicle; N, nucleus. Bar = 1.0 μm.

The characteristics that we used to develop a standardized description of five mammary epithelial cellular morphotypes were: staining of nuclear and cytoplasmic matrix, cell size, cell shape, nuclear morphology, amount and size of cytoplasmic organelles, location within the epithelium, cell number, and grouping relative to each other and to other morphotypes. These characteristics were used to perform differential cell counts and morphometric analysis of the cell populations in rat mammary epithelium (Chepko and Smith, 1997). Table 1 presents a summary of some of the characteristics and functions of each morphotype; the included cartoon of each can be used on both the light and electron levels to help form a search image for recognizing them in situ. The five morphotypes we recognize in rodent mammary epithelium are a primitive small light cell (SLC), an undifferentiated large light cell (ULLC), a very differentiated large light cell (DLLC), the classic cytologically differentiated luminal cell (LDC), and the myoepithelial cell. We described three sets of division-competent cells in rodent mammary epithelium, and demonstrated that mammary epithelial stem cells and their downstream progenitors are morphologically much less differentiated than either the secretory or the myoepithelial cells. We counted a total of 3,552 cells through 17 stages of rat mammary gland development and calculated the percent of each morphotype. This was a retrospective study so the cell count was limited and varied with each stage. Therefore, we chose four of the stages as representative and performed a morphometric analysis using ANOVA and two posteriori tests: Tukey's HSD (Zolman, 1993) and a Bonferoni paired *t*-test to determine the population densities of the two categories of

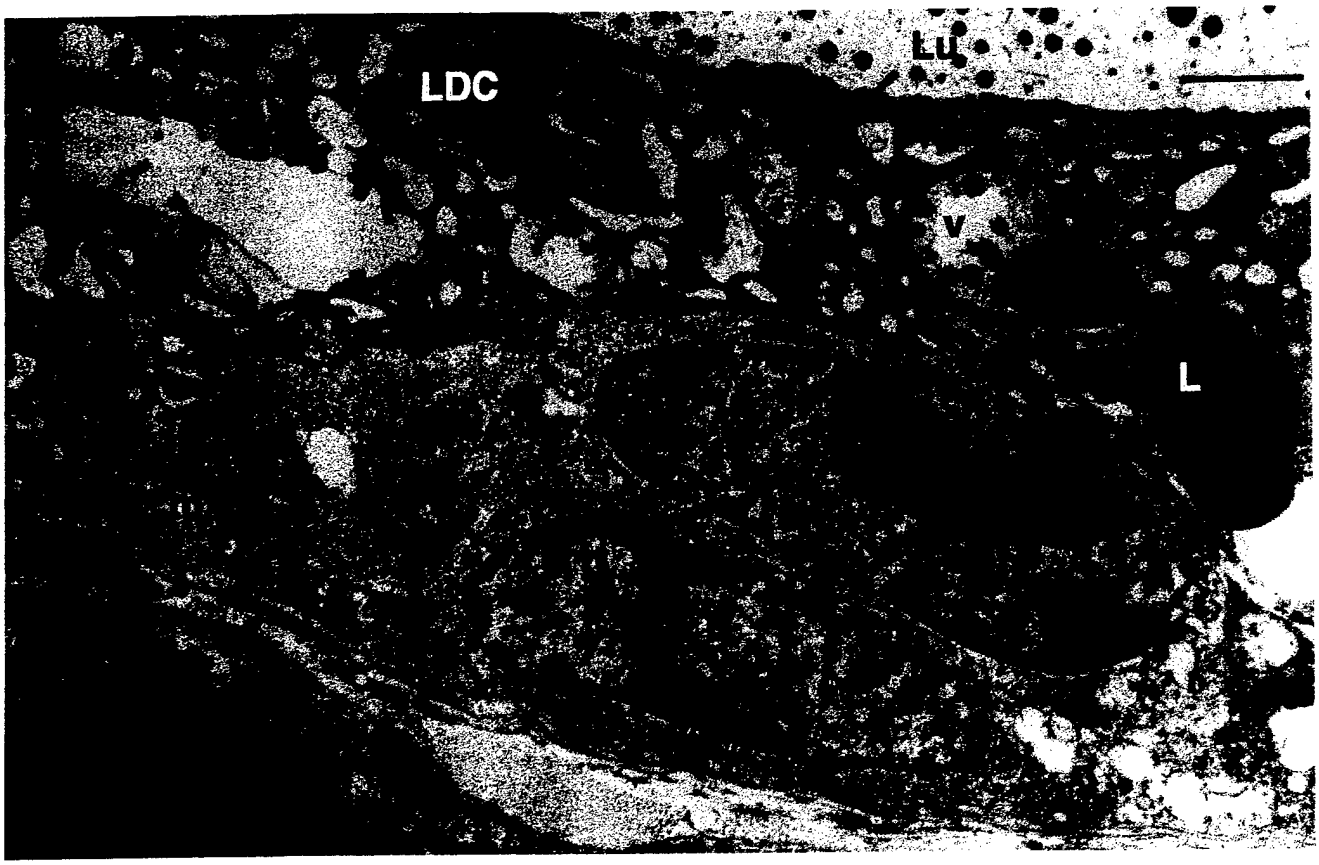

Fig. 4. Transitional characteristics. A one-above-the-other pair of light-staining cells (A and B) with elongated nucleii in 6 day lactating mouse mammary gland. This pairing suggests that an asymmetric mitosis occurred in the recent past. The limit of the plasma membrane of B is indicated by the arrowheads, and that of C by the full arrows. C designates an extension of cytoplasm from another light-staining cell. A is a larger cell than B, and contains well-developed mitochondria (m), as does the cytoplasm of C. B has a small cytoplasm to nuclear ratio indicating that is probably an SLC. The elongated nucleii are characteristic of myoepithelial cells and SLC (see Fig. 1). Bar = 1.0 μm.

light cells (Chepko and Smith, 1997). This analysis showed that the population density (number of cells/mm^2) of SLC did not change from nulliparity through involution. The proportion of SLC remained at 3%. This means that although the number of mammary epithelial cells increased by 27-fold during pregnancy in the mouse (Kordon and Smith, 1998; Nicoll and Tucker, 1965), the percent of SLC in the population does not change. Therefore, SLC increase and decrease in absolute number at the same relative rate as the differentiating epithelial cells. If the SLC were purely a primary stem cell population, they might be expected to show a decrease in population density during epithelial growth, and a concomitant increase with mammary involution. Primary organ-specific stem cells are not expected to behave in this way during tissue remodeling. Therefore, the growth-related fluctuation of SLCs (which are defined solely by morphological criteria) indicates that they more likely represent a combined population of primary stem cells and lineage-committed progenitor cells. If these multipotent cells are morphologically indistinguishable in mammary epithelia, then the constancy of the population density of SLC in the rat mammary gland most likely is due to amplification of primary progenitors. An alternate possibility is that SLC represent primary mammary epithelial-specific stem cells and are maintained proportionally throughout all stages of mammary growth and function for reasons that we do not yet understand.

Multiple images of the five morphotypes listed above are accessible on the Internet at http://mammary.nih.gov/reviews/Chepko001/index.html A brief recapitulation follows.

Small Light Cells

SLC sometimes contain mitotic chromosomes and are, therefore, division-competent. They have a basal location in the epithelium and never touch the lumen. Both the nucleoplasm and cytoplasm are characteristically pale staining, and the nucleus contains dense clumps of heterochromatin and is sometimes indented (Figs. 1 and 2). Organelles are small and show no structural evidence of specialized function. SLC are present in (1) side-by-side homogeneous or like pairs (Fig. 2), (2) heterogeneous or mixed pairs (Fig. 3), (3) in mouse in one-above-the-other pairs (Fig. 4), (4) in clusters with ULLC in every stage of postnatal development, and (5) in mouse mammary explants and hyperplastic alveolar outgrowths (Fig. 5). The pairings are evidence that SLC engage in asymmetric mitosis (sym-

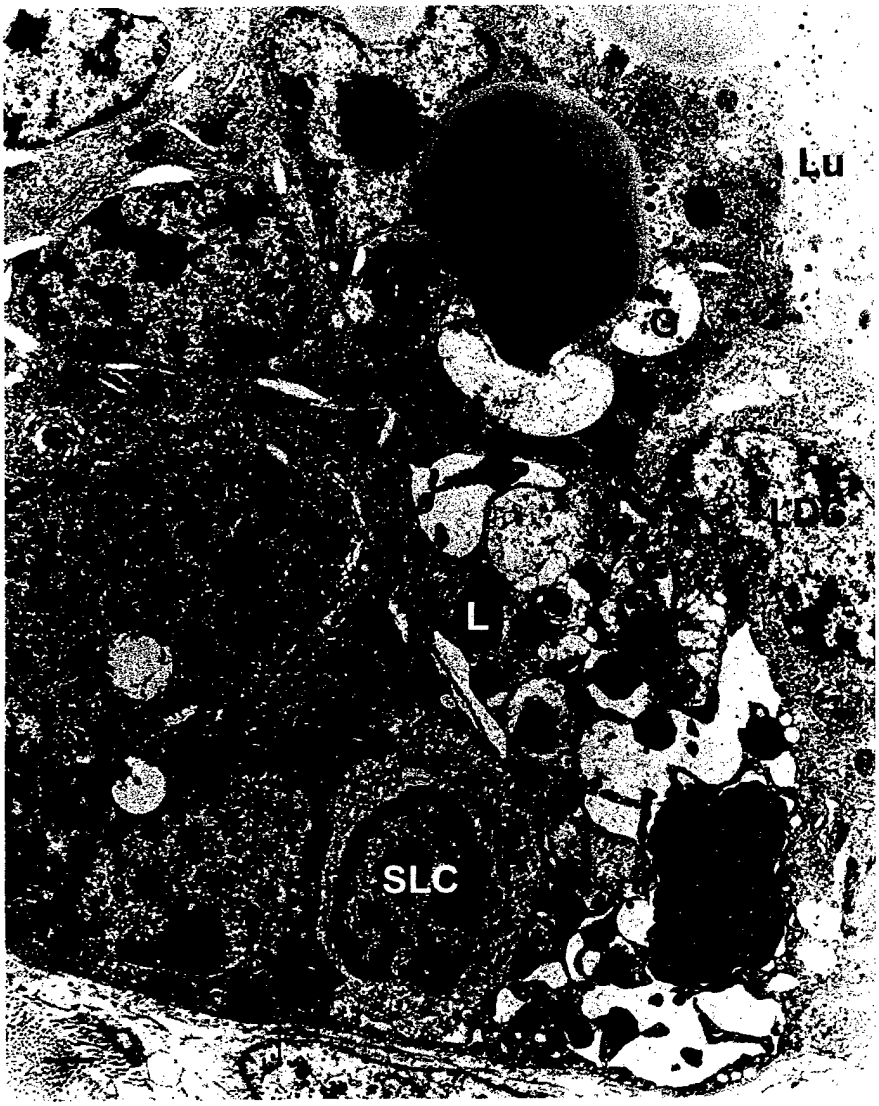

Fig. 5. SLC in hyperplastic mammary pithelium. An SLC is prominent proximal to the basement membrane in this section through a mouse mammary preneoplastic lesion in a hyperplastic alveolar outgrowth from a transplanted fragment. Lu , Lumen; L, Lipid droplet; G, Golgi vesicle; S, secretory cell. Bar = 1.0 μm.

metric mitosis is not inferred from these images). Morphological evidence that SLC differentiate is seen (Fig. 6) in the SLC with characteristics transitional between the very primitive cell seen in Figure 1 and the ULLC in Figure 3. Also the SLC in Figure 7 contains myofibrils and hemi-desmosomes, both specialized organelles for myoepithelial cells. SLC are usually free of specialized membrane contacts with neighboring cells, but occasionally they form non-junctional focal contacts (Fig. 2) between each other and neighboring dark cells. In very rare cases, SLC (one pair out of 111 SLC observed in the rat) may share desmosomes between each other (not shown). Since stem cells are expected to be a very small (perhaps 0.5–1%), fairly stable population, the larger portion of the SLC in rat mammary gland must represent at least one downstream epithelial cell precursor. SLC are usually interpreted as being myoepithelial cells in H&E sections because they are located among the basal epithelium and never contact the lumen.

Large Light Cells

The Large Light Cells also occur in two sets (Table 1), and are morphologically distinguishable into a cytologically undifferentiated morphotype and a differentiated morphotype, also unimaginatively, but accurately designated Undifferentiated Large Light Cells (ULLC) (Fig. 3) and Differentiated Large Light Cells (DLLC) (Fig. 8). The ULLC sometimes contain mitotic chromosomes (Fig. 9), which indicates they are division-competent, they are found to possess mitotic chromosomes in mammary explant cultures whether or not DNA synthesis has been inhibited by cytosine arabinoside, which suggests they may rest at G2 in the cell cycle (Smith and Medina 1988). In cross-section, they are two to three times larger than SLC (Fig. 3). They sometimes, but not always, contact the lumen. Some ULLC that do not contact the lumen have an apically located nucleus and a trailing "tail," that makes them gourd-shaped (Fig. 3). Others, including those that do contact the lumen, are very round cells. ULLC nuclei

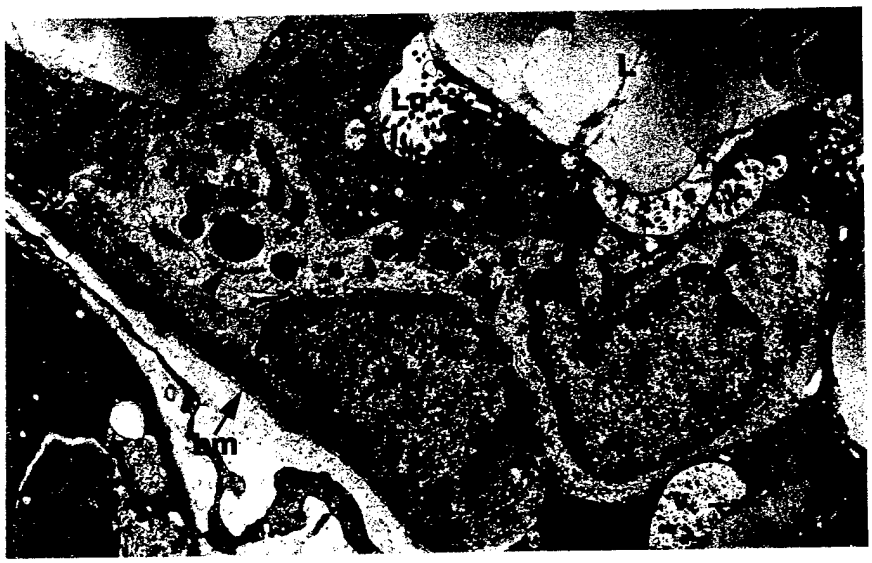

Fig. 6. Transitional characteristics. This cell has features of both SLC and ULLC (Fig. 3). It does not reach the lumen, has a heterochromatic nucleus (N), pale-staining cytoplasm, a little RER (arrowheads), and a small Golgi apparatus (g), and some lipid droplets (L). There is no gap between this cell and its neighbors, and the mitochondria (m) are larger and darker than in Figure 2. The nucleus is located apically (toward the lumen, Lu), as in the ULLC in Figure 3, instead of centrally as in the other SLC shown (Figs. 1, 3). It also has a basally located "foot" or "trailing tail" containing the organelles. Because of these mixed features and the occurrence of SLC in mixed pairs, we believe that SLC are capable of differentiating into ULLC. My, myoepithelial cell; bm, basement membrane; small arrows, RER; G, Golgi vesicle. Bar = 1.0 μm.

are larger and rounder than those of LDC, DLLC, or SLC and contain a pale staining, fibrillar euchromatic nucleoplasm (Fig. 3). Their cytoplasmic matrix is also pale staining. Their cytoplasmic membrane systems are only slightly more developed than those of SLC. Even so, ULLC may contain small secretory granules in their Golgi and lipid droplets in their cytoplasm. When they do not contact the lumen, ULLC make no cellular junctions with their neighbors, but when they do, they form tight junctions and desmosomes with neighboring cells. They also contain small lipid droplets and protein granules even when they are poised for cytokinesis (Fig. 9A,B). ULLC are present in the alveoli of nulliparous, mid-pregnant, and newly lactating rat mammary glands as (1) scattered single cells, (2) one member of a side-by-side mixed pair consisting of itself and an SLC (Fig. 3), (3) side-by-side like pairs, (4) in one above the other mixed pairs where the lower one contains myofibrils and the upper contains some RER and no myofibrils (mouse)(Chepko and Smith 1997), or (5) in small clusters of ULLC and SLC. ULLC in the human also occur in one-above-the-other pairs with the lower one containing myofilaments (Ferguson, 1988). These pairings show that ULLC engage in both symmetric and asymmetric mitosis. ULLC undergo apoptosis both in the nulliparous epithelium and during involution that is initiated by removal of the young at day 4 postpartum.

Differentiated Large Light Cells

DLLC always contact the lumen and were never seen with mitotic chromosomes in their cytoplasm, although they do label with 3H-thymidine in cultured mammary explants induced to secretory differentiation with hormones (Smith and Vonderhaar, 1981; Smith and Medina, 1988). They are secretory cells that contain all of the differentiated features of a classic mammary epithelial secretory cell, except that the cytoplasm is pale staining. In early lactation, DLLC occur in large arrays in rat mammary epithelium and in mouse mammary explants cultured in insulin, hydrocortisone, and prolactin for 48 hours (Smith and Medina 1988). These arrays disappear in rat in vivo by the sixth day of lactation and in the explants after 72 hours culture in the hormone supplemented medium. Apoptotic cells in numbers comparable to the cells in arrays of DLLC were not observed either in the lactating rat or the explants, so we believe the DLLC differentiate into dark cells. Such arrays (which can occupy up to half of the cells in a cross section through a lobular region) suggest that there was a recent large-scale symmetric mitosis (perhaps of ULLC).

KERATIN EXPRESSION AND MAMMARY STEM CELLS

In the mouse, two keratins whose expression has been associated with proliferation (K14) and hyperproliferation (K6) in renewing epithelia were detected in mammary epithelium (Smith et al., 1990). K6 expression was confined in vivo to a small percentage of luminal epithelial cells in subtending ducts but was more highly represented in the body cells of actively growing termini. K14 expression was found in basally located epithelium in fusiform cells corresponding in location and form to myoepithelium. This pattern of K6 and K14 expression was maintained in the mature nulliparous mouse mammary gland. However, in early pregnancy, numerous K6/K14-positive luminal epithelial cells were found among the cells in the newly formed secretory acini. Subsequently, the numbers of K6 and K14-positive luminal cells were reduced coincident with the cessation of lobular growth, when once again K6 and/or K14-positive cells were only rarely observed in the luminal epithelium. In premalignant outgrowths of mouse mammary epithelium, K6 and K14 expression is increased suggesting an association wth epithelial proliferation. In ductal hyperplasias, the luminal cells express K6 (but not K14) whereas in lobular hyperplasia K6 and K14 are concomitantly expressed in the luminal cells. This divergent pattern of expression K6 and K14 in luminal cells mimics that seen during branching morphogenesis as against lobulogenesis during normal growth.

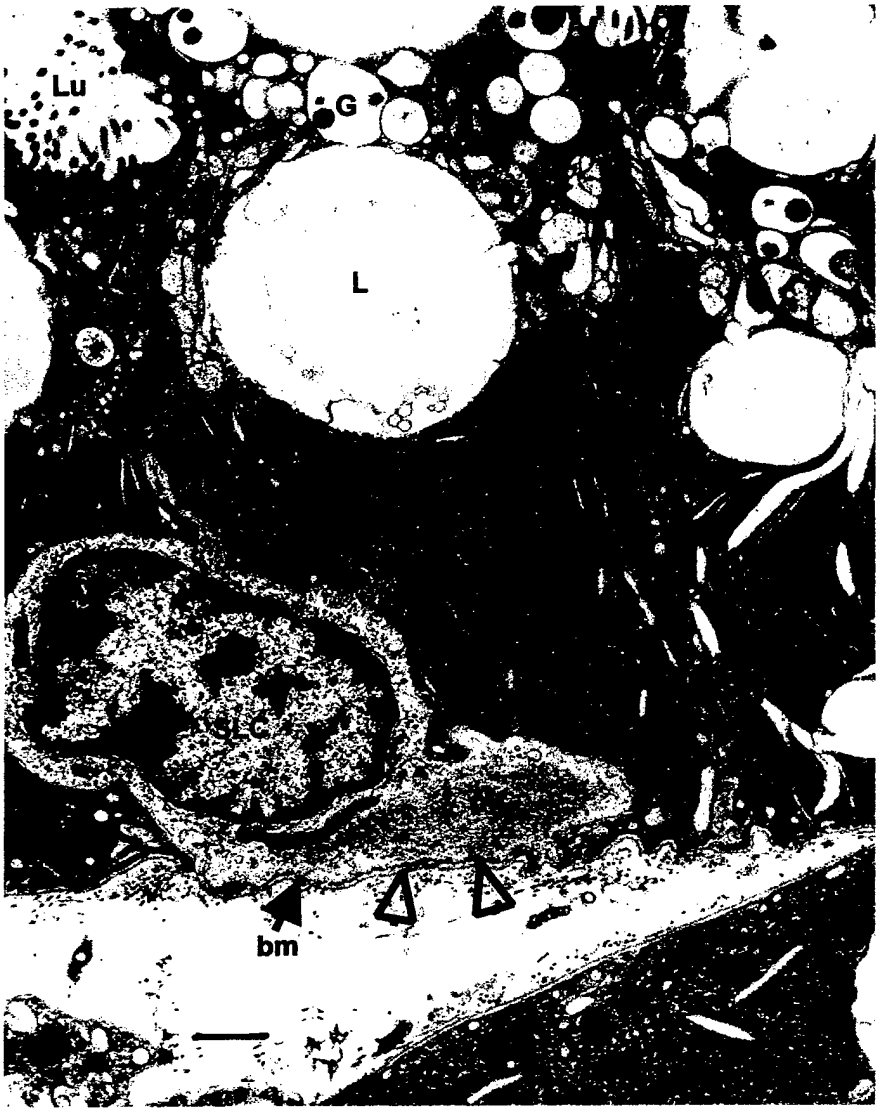

Fig. 7. Transitional characteristics. An SLC in 6-day lactating mouse mammary epithelium. This SLC contains myofibrils (mf) in its basal portion, and is attached to the basement membrane with hemi-desmosomes (arrowheads). Lu, lumen; L, lipid droplet; G, Golgi vesicle; open arrowheads, hemi-desmosomes. Bar = 1.0 μm.

In culture, mouse mammary epithelial cells promiscuously express these cytokeratin markers (K14, K6) (Smalley et al., 1998, 1999; G.H. Smith, unpublished observations). Myoepithelial clones express as expected K14 and smooth muscle α-actin (Smalley et al., 1999), whereas luminal clones express K14, K18, and K19. It is not known whether K6 is also expressed in a subset of cells from these clones. However, I have observed K6 expression in a subset of epithelial cells in primary mammary epithelial cultures after 48 hours of incubation upon plastic substrates (Fig. 10). Keratin 14 is expressed uniformly in all the mammary epithelial cells in these primary cultures (not shown). The location of the K6-expressing cells corresponds with the site of active proliferation in individual epithelial cell colonies. Thus, K6 may be a potential stem cell marker in mouse mammary epithelium. In human mammary epithelial cultures derived from cells expressing CALLA , a myoepithelial cell marker, K14, and α-actin are stably expressed, whereas luminal (MUC-1-sorted) colonies express K18 and K19 (Pechoux et al., 1999). In both mouse and human, only the luminal colonies or clones show the capacity to produce diverse mammary cell phenotypes (Pechoux et al., 1999; Smalley et al., 1999; Stingl et al., 1998). These observations indicate that the pluripotent mammary epithelial cells reside in the luminal cell population in both murine and human mammary glands. This result is in agreement with the location of the undifferentiated cells described above for rodents and pale-staining cells with similar ultrastructure in human breast reported by multiple authors (Ferguson, 1985, 1988; Smith, 1984).

STEM CELL POTENCY AND SENESCENCE

Tissue-specific stem cells appear not to be immortal. Serial transplantation of hematopoetic stem cells (Bortin et al., 1976), and mammary epithelial fragments (Daniel and Young 1971) indicate that stem cells ultimately undergo senescence and stop generating new tissue in host transplantation sites. Mammary epithelial transplants can be carried serially from the cleared fat pad of successive hosts for up to seven generations

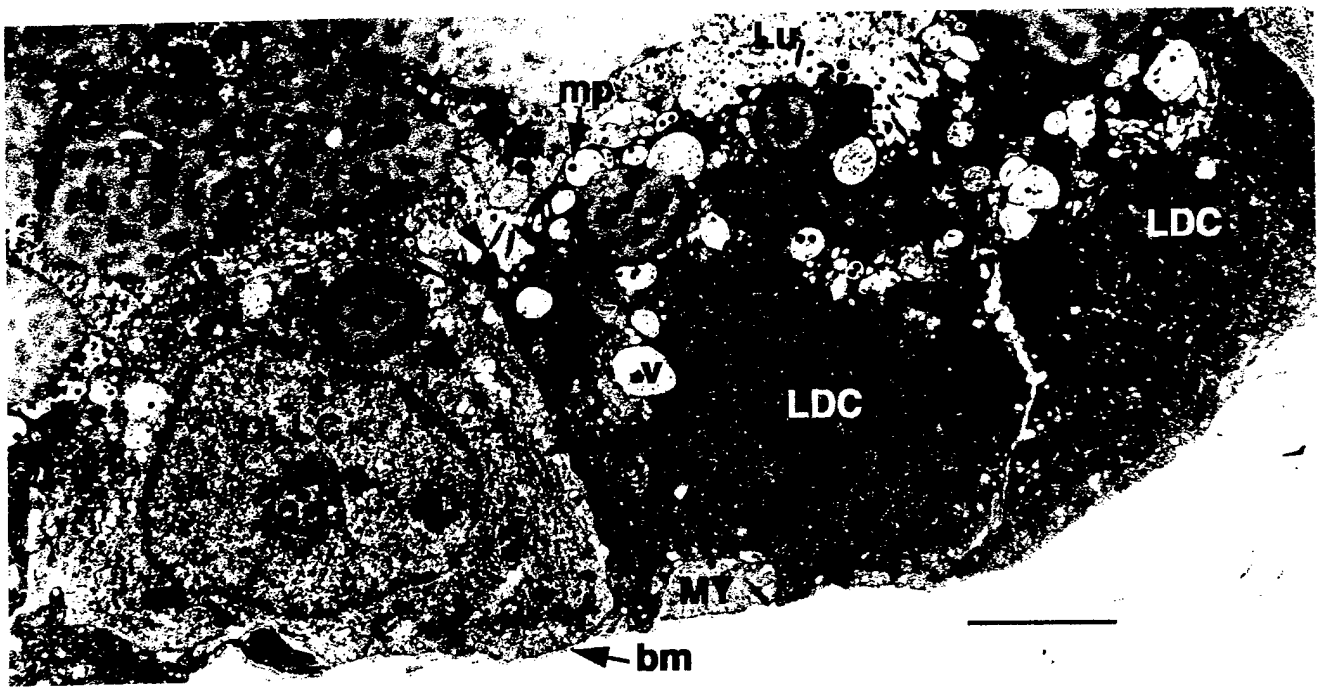

Fig. 8. A differentiated large light cell (DLLC). Rat mammary epithelium at 4 days of lactation. The DLLC has pale cytoplasm and nucleoplasm, but it is otherwise exactly like the active secretory cell beside it (LDC). Both contact the basement membrane and the alveolar lumen. They form tight junctions (T) with neighboring secretory cells, and in pregnant and lactating rats have extensive and organized RER, copious milk protein granules, and large lipid droplets. Unlike ULLC, DLLC are square to polygonal in cross-section. v, Golgi vesicle; L, lipid droplet; bm, basement membrane; Lu, lumen; My, myoepithelial cell; mp, milk protein granule, RER, rough endoplasmic reticulum. Bar = 2.0 μm.

before all portions of a given outgrowth completely lose their capacity to repopulate a mammary fat pad. Greater than 75% of the transplantable fragments show growth arrest at the 4th passage (Daniel et al., 1968). This aging/senescence of the stem cell is associated with a loss of self-renewal capacity and is directly attributable to the number of mitotic events needed to recapitulate tissue replacement (Daniel and Young, 1971; Daniel et al., 1983; Young et al., 1971).

Stem cell populations are difficult to enumerate. However, Cosentino et al. (1996) have shown, using mutation-tagging, that each crypt in the mouse small intestine arises from a single stem cell. The number of multipotent cells residing within an individual crypt at any given time, however, is greater than one as determined by regeneration studies following irradiation (Bach et al., 2000). Further, Tsai et. al., (1996) have demonstrated that normal human mammary epithelium consists of contiguous regions each populated by cells possessing alternate inactivated X-chromosomes. Thus, in the human, individual ducts and their associated lobules are derived from a single stem cell. In practical terms, this means that one stem cell over a lifetime may be the source of thousands of differentiated mammary epithelial cells and of some variable number of multipotent daughters. Transplantation and genetic studies have shown that one stem cell may provide sufficient progeny to completely repopulate mouse mammary gland (Smith, 1996; Kordon and Smith, 1998). Likewise, one stem cell may theoretically suffice for liver (Michalopoulos and DeFrances, 1997) and hematopoetic regeneration (Fraser et al., 1990).

When fragments from senescent mammary outgrowths were transplanted into a host subsequently made pregnant, they remained unable to produce branching ducts, but were capable of developing secretory lobules along their length (Daniel et al., 1971).

This experiment suggests that stem cells may lose their competence to produce progeny that are responsive to signals that induce ductal growth, yet remain sensitive to those that stimulate lobular differentiation in situ. Further, it was demonstrated (Young et al., 1971) that the rate at which fragments of mammary epithelium reached senescence related only to the number of mitotic events that had occurred, and not to the age of the donor or the time allowed for regeneration. This was shown when fragments from the center (point of origin) of an outgrowth were compared to those taken from the periphery of the same outgrowth. The central portions produced larger outgrowths, and continued to produce outgrowths significantly longer than the fragments serially transplanted from the peripheral regions. Recently, one of the authors (G.H.S.) observed that individual fragments from serially-transplanted, genetically-clonal outgrowths were capable of producing a complete ductal tree in pregnant hosts without producing a single secretory lobule. This demonstrates that the capacity of mammary stem cells to give rise to lobular progenitors by asymmetric division was lost independent of the capacity to produce ductal-committed offspring during the process of stem cell senescence. This mechanism may explain the existence of apparently immortal alveolar and ductal-dominant mammary outgrowths that are reproducibly dis-

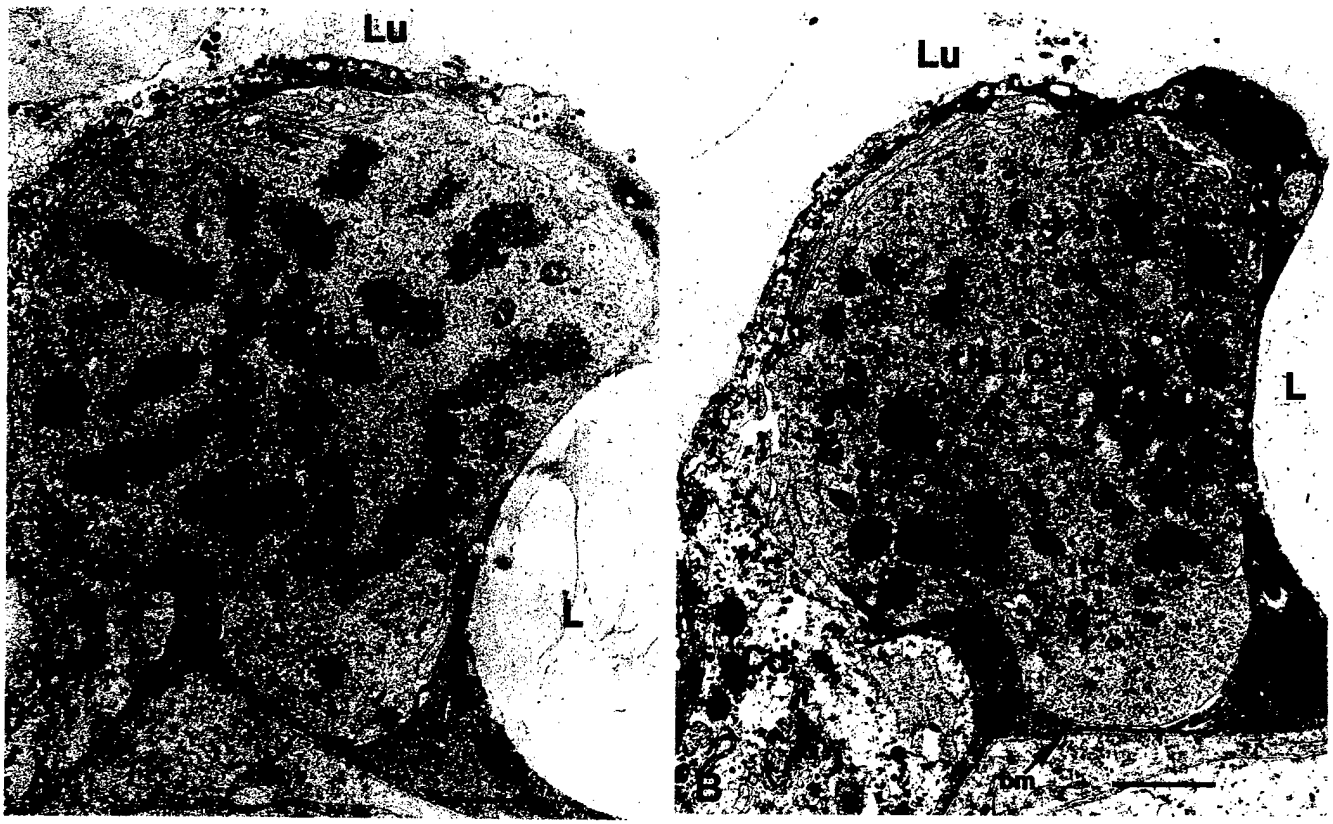

Fig. 9. Symmetric mitosis in ULLC. Two (of seven) serial sections, shown in A and B, through a gourd-shaped ULLC in mitosis in rat mammary epithelium at 2–3 hours lactation. This is symmetric mitosis because the metaphase plate is perpendicular to the basement membrane. These sections show that in the rat, these cells divide below the lumen. In the mouse, they can contact the lumen during mitosis. The presence of numerous lipid droplets and organelles in this mitotic cell shows that cell division and differentiation toward secretory competence occur simultaneously in ULLC. C, condensed chromosomes; L, lipid droplets; Lu, lumen; my, myoepithelial cell; bm, basement membrane; Cd, damaged DLLC; E, capillary endothelial cell. Bar = 1.5 μm.

covered in chemically transformed mammary tissues (Smith et al., 1981).

HOW MANY STEM CELLS?

Allophenic mice produced by fusing the blastomeres from two different inbred strains possess chimeric mammary epithelium containing clonogenic cells from each strain (Mintz and Slemmer, 1969). This suggests that each gland must be derived from the progeny of at least two stem cells. Epithelial fragments transplanted to host fat pads contain a few thousand mammary epithelial cells (Smith and Medina, 1988). Following transplantation into breeding hosts, these fragments produce lactating outgrowths that contain 60 million epithelial cells (Kordon and Smith, 1998). This is an 8,500-fold increase in epithelial cell number, and the number of cell divisions required to produce such outgrowths, if they are derived from a single cell, is theoretically 25 and 27, respectively (average: ~26). Therefore, it seems unlikely that one stem cell would be able to divide symmetrically 25–27 times and still be able to self-renew multipotent progeny sufficient to repopulate the entire gland and maintain these pluripotent offspring through several successive transplant generations, before beginning to show proliferative aging (Daniel and Young, 1971). Transplantation of mouse mammary epithelial cells at limiting dilution (Smith, 1996) indicated the presence of a clonogenic epithelial cell in every 2,500 cells. Similar studies in the rat indicate that clonogenic cells capable of ductal morphogenesis are present at 1:20,000 cells and alveolar clonogens at 1:950 cells among the dispersed epithelial cells from nulliparous female rats (Kamiya et al., 1998). Since the total number of epithelial cells in a nulliparous mouse mammary gland is about 2.5×10^6 (Kordon and Smith, 1998), then the estimated number of stem cells is about 1,000 under steady-state conditions. If complete glands regenerated from a single cell in nulliparous hosts contain about 1,000 stem cells, it would require 11 symmetrical divisions by a lone antecedent to produce the multipotent epithelial subset. After four serial transplants, the original clone-generating stem cell would have been self-renewed between 40 to 50 times, and this fits well with the number of doublings expected from a mortal eukaryotic cell before the onset of senescence (Hayflick, 1992).

SYNOPSIS

Evidence from serial transplantation of mammary epithelial fragments or cells has shown that there are mammary epithelial stem cells, and they may produce lineage-limited progenitors. These oligopotent progenitors appear to be limited in their ability for self-renewal. Ultrastructural evidence supports

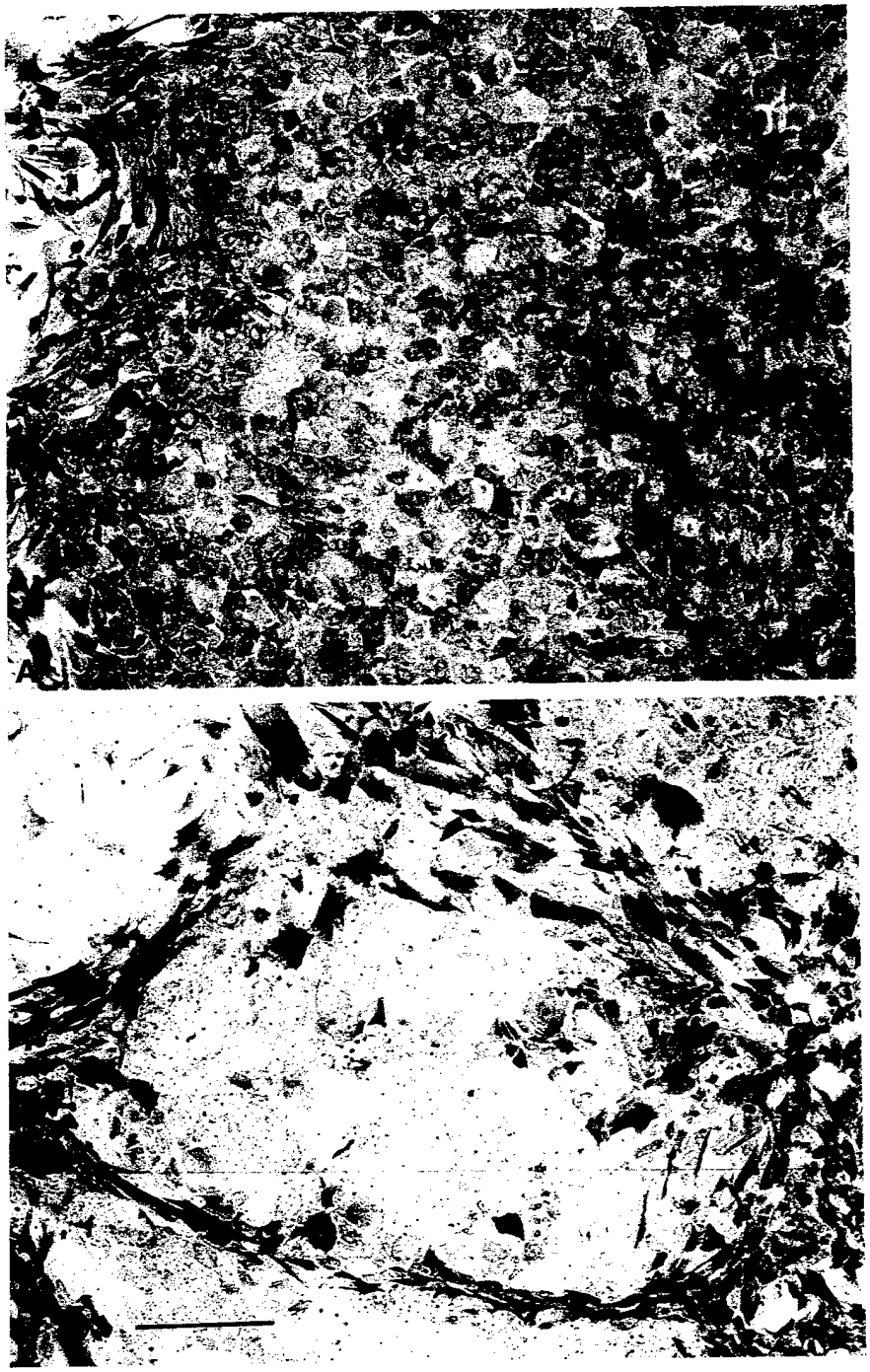

Fig. 10. Mammary epithelial colonies in primary cultures 4 days after plating were reacted with anti-K14 (A) or anti-K6 (B) (Smith et al., 1990). The darkly stained cells are positive for expression. Although nearly all of the epithelial cells were positive for K-14 (A), only a subset of these expressed. K6. Bar = 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the notion that there are three division-competent cells and that two of them, the SLC and the ULLC, can produce myoepithelial cells. Evidence from in vitro studies, both in mouse and human, demonstrates that the mammary luminal epithelial population contains the multipotent epithelium. This suggests that the more luminal ULLC may represent the active functional mammary stem cell whereas the more basal SLC may represent the quiescent form.

SLC and ULLC are present in vigorously growing normal mammary transplant outgrowths as well as in premalignant alveolar outgrowths. SLC and ULLC are not present in epithelial fragments that produce senescent outgrowths following serial transplantation into gland-free fat pads (Smith, unpublished data). This result provides strong morphological support for the conclusion that SLC and ULLC represent the major morphological manifestation of competent mammary epithelial stem cells.

Both SLC and ULLC have been identified in the mammary epithelium of every mammal that we have examined: rat, mouse, human, (Chepko and Smith 1997) as well as in sheep (Ellis, 1995), and cow (Ellis, personal communication). They are also present in greater than normal numbers in some human and mouse mammary tumors (Smith and Chepko, unpublished data). This observation implicates both SLC and ULLC morphotypes in the origin of mammary cancer.

Although it is generally accepted that 3H-thymidine or 5-BrDU incorporation (Traurig, 1967; Zeps et al., 1998) is evidence of mammary cell proliferation, the cells in explants cultured from mammary glands that were found in mitosis, primarily ULLC and SLC, were not the cells that incorporated 3H-thymidine (Smith and Medina 1988) during the culture period. The percentage of mammary epithelial cells that incorporated 3H-thymidine in explant cultures from nulliparous mice was compared with the number that entered mitosis (Smith and Vonderhaar, 1981; Vonderhaar and Smith, 1982). The explants were cultured in medium containing insulin, hydrocortisone, prolactin, 3H-thymidine, and colchicine for 72 hours followed by a 12-hour chase in cold thymidine. Fifty to 55% of the luminal mammary epithelial cells in replicate cultures incorporated 3H-thymidine into their nuclei during the culture period, but mitotic figures were found in only 5%. Nevertheless, after 72 hours, 30% of the epithelial cells were immunopositive for casein, an increase of approximately 10-fold over the number in zero time explants. This increase is much greater than can be accounted for by the number of mitotic events that were observed. Some of the mitotic cells in these cultures were immunopositive for casein, indicating their response to lactogenic stimuli. Nevertheless, DNA synthesis is required in nulliparous (but not primiparous) mouse mammary explants for a lactogenic response but not for mitosis (Smith and Medina 1988; Smith and Vonderhaar, 1981; Vonderhaar and Smith, 1982). It is also important to note that a similar dependency upon extracellular proline in explants from nulliparous mouse mammary glands (Smith, 1987) for the hormonal induction of a proliferative and lactogenic response was absent in the epithelium of explants from age-matched primiparous donors. These observations demonstrate a marked difference in the response of primiparous and nulliparous mammary epithelial populations in vitro to lactogenic hormones. No difference was noted in the response of the SLC or ULLC to lactogenic hormones in explants from primiparous vs. nulliparous females. This suggests that the progeny of multipotent mammary stem cells following a single pregnancy are different from those present in the nulliparous gland. An early pregnancy is protective with respect to lifetime risk for the development of breast cancer in humans and in rodents (Medina and Smith, 1999). These observations indicate that we do not have a clear understanding of the relationship between DNA synthesis, epithelial cell proliferation, and functional differentiation in mammary epithelium. Therefore, the study of mammary epithelial stem cells and the signals that regulate their behavior in vivo is vital to our understanding the mechanisms of neoplastic transformation in mammary epithelium. At present, there are no specific proven cellular markers that unequivocally identify one mammary epithelial cell from another as representative of its ability to perform stem cell functions.

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